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13. ABSTRACT *(Maximum 200 Words)*

We have previously shown significantly increased Akt kinase activity and AKT2 protein level in primary ovarian carcinoma. Recently, we have demonstrated that activation and overexpression of Akt contribute to chemoresistance by 1) phosphorylation of ASK1 leading to inhibition of JNK and p38 kinase activity, 2) induction of XIAP through a interaction/phosphorylation mechanism and 3) phosphorylation of p21 leading to G2/M progression. In addition, we have also demonstrated that inhibition of PI3K/Akt pathway by PI3K inhibitor and farnesyltransferase inhibitor (FTI) sensitizes ovarian cancer cells to cisplatin-induced apoptosis. These data indicate that Akt is a critical target for ovarian cancer intervention and that inhibitor(s) of PI3K/Akt pathway and FTI may improve the outcome of ovarian cancer.

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Introduction

The purpose of this project is to: 1) Determine the incidence and clinical significance of *PI3K/AKT1* alterations in ovarian cancer; 2) Determine the role of overexpression of active and wild type PI3K and AKT1 in ovarian surface epithelial cell transformation and 3) Determine PI3K and AKT1 as targets for ovarian cancer intervention.

Body:

During the last budget year, we have determined biological significance of activation/overexpression of PI3K/Akt pathway in human ovarian cancer and examined the PI3K and Akt as targets for ovarian cancer intervention.

1. PI3K/Akt activation contributes to chemoresistance.

We have previously shown significant increase of Akt kinase activity and AKT2 protein levels in primary ovarian carcinomas (1, 2). To determine biological significance of PI3K/Akt alterations in human ovarian cancer, cisplatin sensitive (A2780-S and OV2008) and resistance (A2780-CP and C-13) ovarian cancer cell lines were stably transfected with DN-Akt and constitutively active Akt (Myr-Akt), respectively. Cell growth and Tunel assay analyses revealed that Myr-Akt transfected A2780-S and OV2008 became resistant to cisplatin, whereas DN-Akt sensitized A2780-CP and C-13 cells to cisplatin-inhibited tumor cell growth (Fig. 1 and data not shown).

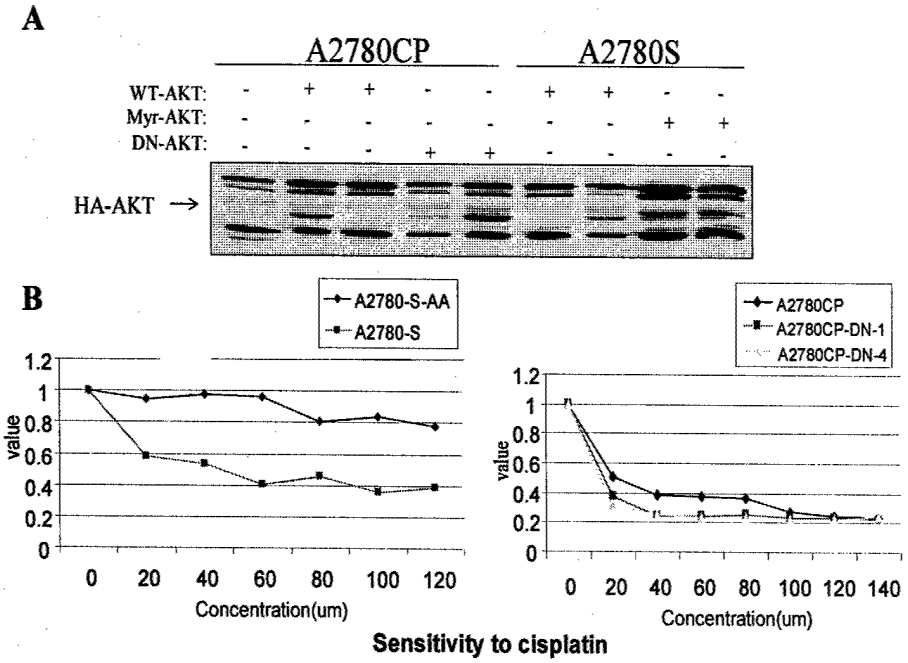

Fig. 1. Activation of Akt in ovarian cancer cells contributes to chemoresistance. (A) Western blotting analyses of AKT expression. Cisplatin resistance (A2780-CP) and sensitive (A2780-S) cell lines were stably transfected with HA-tagged dominant negative (DN) AKT and constitutively active AKT (Myr-AKT), respectively. Cell lysates from clonal cell lines were subjected to immunoblotting analyses with anti-HA antibody. (B) Clonal cell lines were treated with indicated concentration of cisplatin for 36 h. Cell viability was examined with MTS assay. Constitutively active AKT (AA) transfected A2780-S became resistance to cisplatin (left), whereas dominant negative AKT transfected A2780-CP cells (both clone 1 and 4 cell lines) sensitize to cisplatin treatment as compared to parental cells (right).

We have also established pTet-on/pTRE2-HA-DN-*Akt* and –HA-myr-*Akt* stably-transfected, i.e. doxycyclin inducible, Hela clonal cell lines. Two (clones 6 and 13) of 18 DN-*Akt* and 1 (clone 27) of 18 myr-*Akt* clonal lines are not leaky (Fig. 2A and B). After induction with doxycycline (1 μg/ml), DN-Akt expresses at very high level, blocks EGF-induced kinase activity of 3 isoforms of endogenous Akt (Fig. 2C), and inhibits cell growth (36% less than that in uninduced cells, Fig. 2D). These clonal cell lines were treated with commonly used chemotherapeutic drugs, including cisplatin, paclitaxel, etopside, vincristine, metrotrexate, and doxorubicin. Tetrazolium salt microtiter plate assay (CellTiter 96 Cell Proliferation Assay, Promega) revealed that the cells expressing constitutive activated Akt became to resistant to paclitaxel, etopside, and cisplatin (Fig 2E) whereas DN-Akt sensitized the cells to some chemotherapeutic reagent-induce cell death (Fig. 2F). These data indicate that activation of Akt in ovarian cancer contributes to chemoresistance and that PI3K/Akt pathway is a critical target for ovarian cancer intervention.

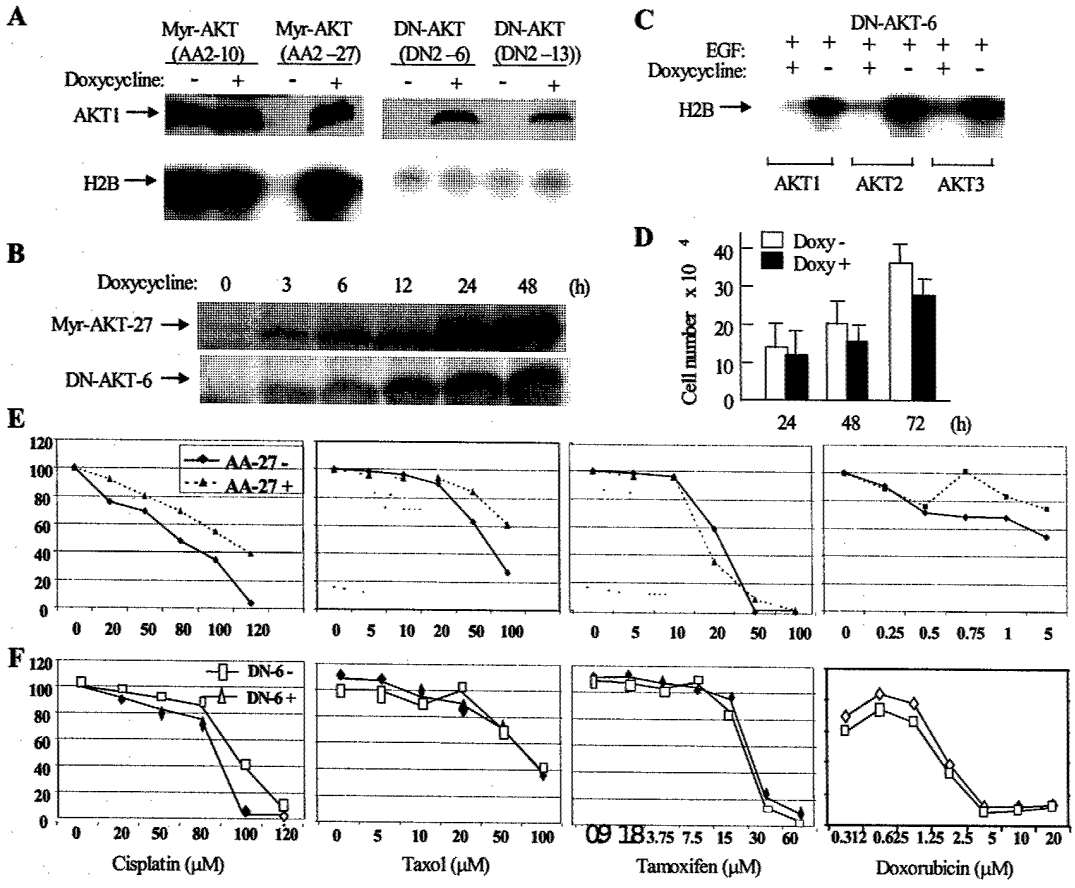

Fig. 2. *Inducible expression of HA-myr-AKT and HA-DN-AKT in Hela-derived cell lines, which are related to chemotherapeutic drug sensitivity. **(A)** Western blot (top panel) and in vitro kinase (bottom panel) analyses of Myr-AKT (clones 10 and 27) and DN-AKT (clones 6 and 13) clonal cell lines. Cells were treated with (+) or without (-) doxycycline for 48 h. **(B)** Myr-AKT-27 and DN-AKT-6 cells were exposed to doxycycline for indicated times. Cell extracts were immunoblotted with anti-HA antibody. **(C)** In vitro kinase assay of endogenous AKT1, AKT and AKT3 immunoprecipitates from DN-AKT1-6 cells stimulated with EGF for15 min after treatment with or without doxycycline (1 μg/ml) for 48 h. **(D)** Cell growth in DN-AKT1-6 clonal cell line treated with or without doxycycline (1 μg/ml) for indicated times. **(E)** Hela cells expressing myr-AKT became resistant to the indicated drugs. **(F)** Hela cells exprssing DN-AKT are sensitized to cisplatin.*

2. PI3K/Akt antiapoptotic function is mediated by regulation of XIAP and ASK1-JNK/p38 pathway.

Recent studies demonstrated that the X-linked inhibitor of apoptosis protein (Xiap) plays a key role in cell survival by modulating death signaling pathways and is a determinant of cisplatin resistance in ovarian cancer cells (3, 4). We have examined if Akt contributes to chemoresistance by regulation of XIAP. Coimmunoprecipitation and Western blot analyses showed that Akt binds to and stabilizes XIAP. Ectopic expression of constitutively active Akt phosphorylates XIAP *in vitro* and *in vivo* and inhibits cisplatin and taxol-induced XIAP ubiquitination and degradation (4, see appendix).

It has been well documented that JNK and p38 MAPKs are required for chemotherapeutic drug- and cellular stress-induced apoptosis (5-8). To determine if PI3K/Akt induced cell survival is mediated by JNK/p38 pathway, we have examined the effects of PI3K/Akt pathway on DNA damage-induced JNK/p38 activation. Our results showed that cisplatin and TNFα significantly induced kinase activities of JNK and p38 in human A2780 ovarian cancer cells (9, see appendix). Expression of constitutively active PI3K, p100*, and Myr-Akt abrogates JNK/p38 activation induced by cisplatin and TNFα, indicating that cisplatin resistance in human ovarian cancer cells with high levels of PI3K and Akt is in some extent mediated by PI3K/Akt inhibition of cisplatin-stimulated JNK/p38 activation. To further define the mechanism of PI3K/Akt inhibition of JNK/p38, we have examined the effect of PI3K/Akt on ASK1 kinase activity, which is a key molecule to activate JNK/p38. We have observed that Akt interacts with and inhibits ASK1 by phosphorylation of Serine-83 of ASK1. Expression of nonphosphoryable ASK1-S83A sensitized A2780 ovarian cancer cells to cisplatin-induced apoptosis and abrogated the inhibitory effects of Akt on JNK/p38 activation, whereas phosphorylation mimic ASK1-S83D rendered cells to resistant to programmed cell death. These results indicate that activated PI3K/Akt protection of cells from cisplatin-induced apoptosis is mediated by Akt inhibition of stress kinases and provide the evidence that Akt inhibition of JNK/p38 is via inhibition of ASK1 kinase.

3. Akt overrides DNA damage-induced G2-M arrest and contributes to chemoresistance through phosphorylation of $p21^{Cip1/WAF1}$

We have observed that Akt phosphorylation of p21 overrode G2/M arrest induced by γ-irradiation and chemotherapeutic agents such as texol and cisplatin. Akt bound to and phosphorylated p21 on threonine-145 both *in vitro* and *in vivo*. Cisplatin-sensitive A2780-S and HCT116 cells expressing p21T145D, converting Akt phosphorylation site to aspartic acid, became resistant to cisplatin-induced apoptosis, texol- and γ-irradiation-induced G2/M arrest, whereas expression of p21T145A form, changing threonine-145 into alanine, sensitized cells to apoptosis and abrogated Akt-induced cell survival. These data indicate that p21 is an important physiological substrate of Akt and that Akt phosphorylation of p21 may play a pivotal role in Akt-regulated cell cycle and cell survival and could contribute to drug resistance.

4. PI3K inhibitor and FTI sensitize ovarian cancer cells to apoptosis.

To determine if inhibition of PI3K/Akt pathway sensitizes chemotherapeutic drug-induced programmed cell death in human ovarian cancer cells, cisplatin resistance A2780-CP and C13 cells were treated with cisplatin together with or without PI3K inhibitor LY294002 and farnesyltransferase inhibitor (FTI), which has been shown by us to specifically inhibit PI3K/Akt pathway (10). Apoptosis was significantly induced by treating the cells with cisplatin/LY294002 or cisplatin/FTI as compared to cisplatin alone. These data indicate that PI3K/Akt pathway is a critical target for ovarian cancer intervention. The reagents targeting PI3K or Akt could be potential drugs for ovarian cancer treatment, especially in chemoresistant tumors.

Key Research Accomplishment

1 Activation of PI3K/Akt in human ovarian cancer contributes to chemoresistance.
2 XIAP and ASK1-JNK mediate PI3K/Akt antiapoptotic function.
3 Akt phosphorylates p21 to override DNA damage-induced G2-M arrest.
4 Inhibition of PI3K/Akt pathway by PI3K inhibitor and FTI sensitizes cisplatin resistant ovarian cancer cells to apoptosis.

Reportable Outcomes

1. Inhibition of JNK by cellular stress- and tumor necrosis factor α-induced AKT through activation of the NFκB pathway in human epithelial Cells. J Biol Chem. 277(33):29973-2982, 2002.
2. Apoptosis Protein in chemoresistance in Ovarian Cancer: Possible involvement of the PI 3-Kinase/Akt Pathway. Drug Resist Updat. 5:131-146, 2002.
3. PI3K/AKT pathway regulates TSC tumor suppressor complex by phosphorylation of tuberin. *J. Biol. Chem.* 277:35364-35370, 2002.
4. Positive feedback regulation between Akt and MyoD during muscle differentiation. Cloning of Akt promoter. J Biol Chem. 277:23230-23235, 2002.
5. Telomerase is regulated by JNK in ovarian surface epithelial cells. *Cancer Res.,* 62:4575-4578, 2002.

Conclusion

1. PI3K/Akt pathway plays a pivotal role in ovarian cancer chemoresistance.
2. Akt inhibition of JNK/p38 by phosphorylation of ASK1 and Akt stabilization of XIAP mediate Akt antiapoptotic function.
3. Akt regulates G2/M cell cycle progression through phosphorylation of p21.
4. PI3K and Akt are critical targets for ovarian cancer intervention.

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Appendices

1 Yuan ZQ, Feldman RI, Sun M, Olashaw NE, Coppola D, Sussman GE, Shelley SA, Nicosia SV, Cheng JQ. Inhibition of JNK by cellular stress- and TNFa-induced AKT through activation of the NFκB pathway in human epithelial cells. *J Biol Chem.* 277:29973-29982, 2002.

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Inhibition of JNK by Cellular Stress- and Tumor Necrosis Factor α-induced AKT2 through Activation of the NFκB Pathway in Human Epithelial Cells*

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Previous studies have demonstrated that AKT1 and AKT3 are activated by heat shock and oxidative stress via both phosphatidylinositol 3-kinase-dependent and -independent pathways. However, the activation and role of AKT2 in the stress response have not been fully elucidated. In this study, we show that AKT2 in epithelial cells is activated by UV-C irradiation, heat shock, and hyperosmolarity as well as by tumor necrosis factor α (TNFα) through a phosphatidylinositol 3-kinase-dependent pathway. The activation of AKT2 inhibits UV- and TNFα-induced c-Jun N-terminal kinase (JNK) and p38 activities that have been shown to be required for stress- and TNFα-induced programmed cell death. Moreover, AKT2 interacts with and phosphorylates IκB kinase α. The phosphorylation of IκB kinase α and activation of NFκB mediates AKT2 inhibition of JNK but not p38. Furthermore, phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 significantly enhances UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibits programmed cell death in response to UV and TNFα stimulation with an accompanying decreased JNK and p38 activity. These results indicate that activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence that AKT inhibits stress kinase JNK through activation of the NFκB pathway.

Exposure of cells to environmental stress results in the activation of several signal transduction pathways including the MEKK4/MKK7/JNK,[1] MKK3/MKK6/p38, and IκB kinase (IKK)/IκB/NFκB cascades. Stress-induced clustering and internalization of cell surface receptors, such as those for platelet-derived growth factor, tumor necrosis factor α (TNFα), epidermal growth factor, and insulin-like growth factor 1 (IGF1), mediate stress-kinase activation (1–3). Recent studies suggest that nearly all stress stimuli activate phosphatidylinositol 3-kinase (PI3K) (1), and of the downstream targets of PI3K, AKT is thought to play an essential role in the cellular response to stress.

AKT, also termed protein kinase B or RAC kinase, represents a family of PI3K-regulated serine/threonine kinases (4, 5). Three different isoforms of AKT have been identified, AKT1/protein kinase Bα (AKT1), AKT2/protein kinase Bβ (AKT2), and AKT3/protein kinase Bγ (AKT3), all of which are activated by growth factors in a PI3K-dependent manner (4–9). Full activation of the AKTs requires their phosphorylation at Thr^{308} (AKT1), Thr^{309} (AKT2), or Thr^{305} (AKT3) in the activation loop and Ser^{473} (AKT1), Ser^{474} (AKT2), or Ser^{472} (AKT3) in the C-terminal activation domain (9). AKT1, the most studied isoform, which was originally designated as AKT, suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. Possible mechanisms by which AKT1 promotes cell survival include phosphorylation and inactivation of the proapoptotic proteins BAD and caspase-9 (10, 11). AKT1 also phosphorylates and inactivates the Forkhead transcription factors, an event that results in the reduced expression of the cell cycle inhibitor, $p27^{Kip1}$, and the Fas ligand (12–14). Via phosphorylation of IKK, AKT1 also activates NFκB, a transcription factor that has been implicated in cell survival (15, 16).

Two separate studies demonstrated that AKT1 is activated when NIH 3T3 fibroblasts are stressed in a variety of ways (17, 18). Based on data showing that PI3K inhibitors do not prevent AKT1 activation by stress, these studies concluded that stress-induced AKT1 activation was PI3K-independent. Other studies, however, found that PI3K activity was required for AKT1 activation by heat shock or oxidative stress in Swiss 3T3 cells (19, 20). It has been suggested that certain cellular stresses activate AKT1 and AKT3 but not AKT2 (19), a finding that is consistent with the different functions of the AKTs as revealed by studies of mice lacking AKT1 or AKT2 (21–23). Nevertheless, activation of AKT2 by stress and the role of AKT2 in the stress response have yet to be fully explored. The data presented here show that AKT2 is significantly activated by stress stimuli (*e.g.* UV irradiation, heat shock, and hyperosmolarity) and by TNFα in human epithelial cells but not in fibroblasts. Stress-induced AKT2 activation in epithelial cells is completely blocked by inhibitors of PI3K. When activated by stress, AKT2 inhibits JNK and p38 activities that are required for stress-induced apoptosis. In addition, AKT2 binds to and phosphoryl-

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[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; TNFα, tumor necrosis factor α; IGF1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; IKK, IκB kinase; NIK, NFκB-inducing kinase; GST, glutathione *S*-transferase; HEK, human embryonic kidney; TUNEL assay, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay.

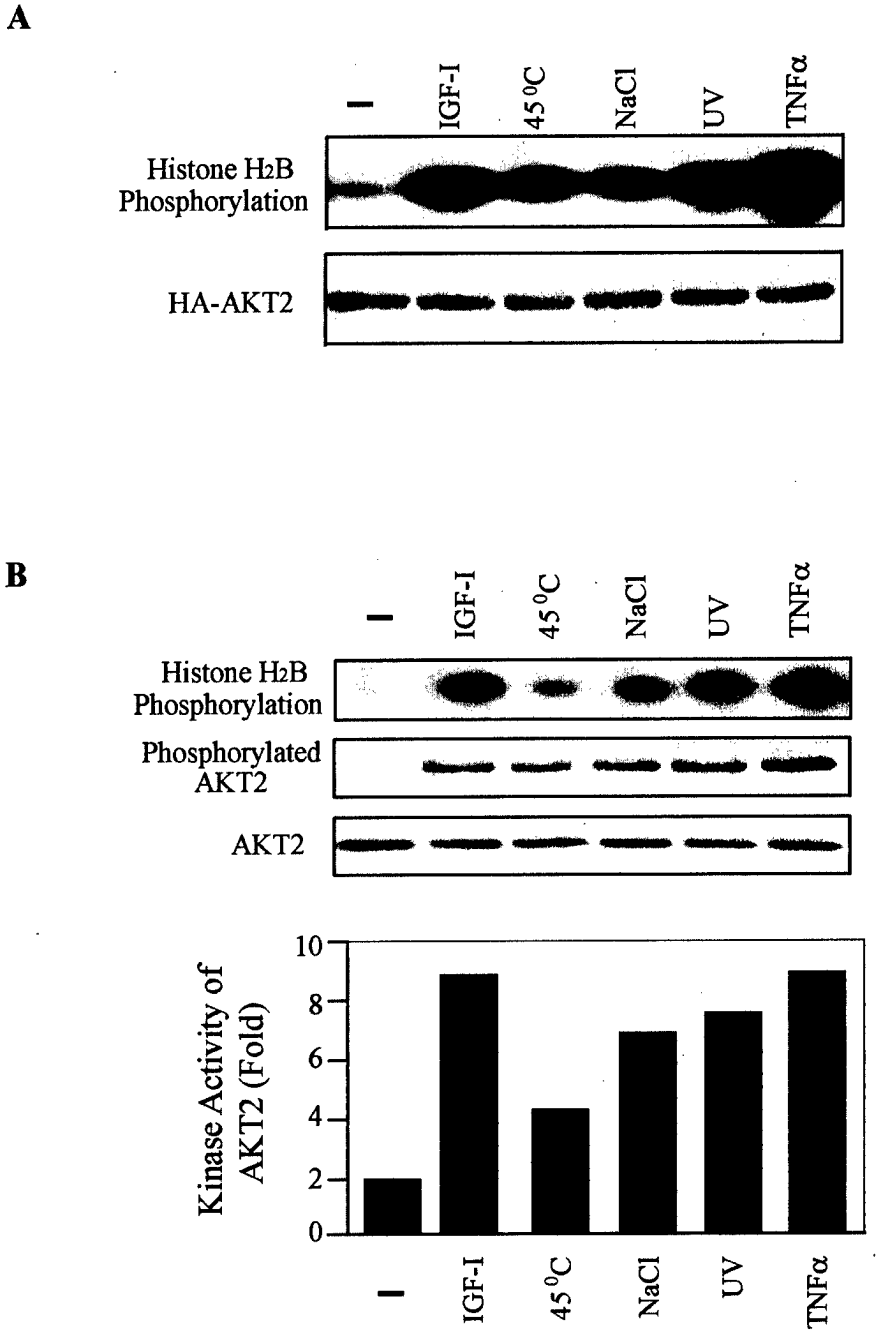


FIG. 1. **AKT2 is activated by cellular stress and TNFα.** *A*, *in vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transiently transfected with HA-AKT2. Cells were exposed to 100 ng/ml IGF-1 (15 min), heat shock (45 °C for 20 min), 0.4 M NaCl (15 min), 40 J/m^2 UV-C (254 nm), or TNFα 20 ng/ml (15 min), and AKT2 activity was determined by *in vitro* kinase assay using histone H2B as substrate. *B*, OVCAR3 cells were treated with the indicated stimuli and immunoprecipitated with anti-AKT2 antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (*upper*) and Western blotting analyses with anti-phospho-Ser473 AKT (*middle*), or anti-AKT2 (*lower*) antibody. The *bottom panel* shows relative AKT2 kinase activity quantified by phosphorimaging. Each experiment was repeated three times.

ates IKKα and, consequently, activates NFκB, resulting in inhibition of programmed cell death in response to stress stimuli. Moreover, AKT2-induced NFκB activation is required for the inhibition of JNK, but not p38, activity.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Stimulation—The human epithelial cancer cell lines, A2780, OVCAR3, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells/dish. After incubation overnight, the cells were transfected with 2 μg of DNA/dish using LipofectAMINE Plus (Invitrogen). After 36 h of the transfection, the cells were serum-starved overnight and stimulated with UV-C irradiation, heat (45 °C), 0.4 M NaCl, or 20–50 ng/ml TNFα.

Expression Constructs—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2, constitutively active HA-Myr-AKT2, and dominant negative HA-E299K-AKT2 have been described (24). The HA-JNK1 construct was kindly provided by Michael Karin (School of Medicine, University of California at San Diego). GST-c-Jun-(1–79) and pCMV-FLAG-p38 were gifts from Roger J. Davis (School of Medicine, University of Massachusetts). The constructs used in the study of the NFκB pathway were prepared as previously described (25).

Immunoprecipitation and Immunoblotting—Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4 °C before immunoprecipitation or Western blotting. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. After the removal of the beads by centrifugation, lysates were incubated with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals), anti-FLAG antibody (Sigma), or anti-AKT2 antibody (Santa Cruz Biotechnology) in the presence of 30 μl of protein A-protein G (2:1)-agarose beads for 2 h at 4 °C. The beads were washed once with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10, twice with phosphate-buffered saline, and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10

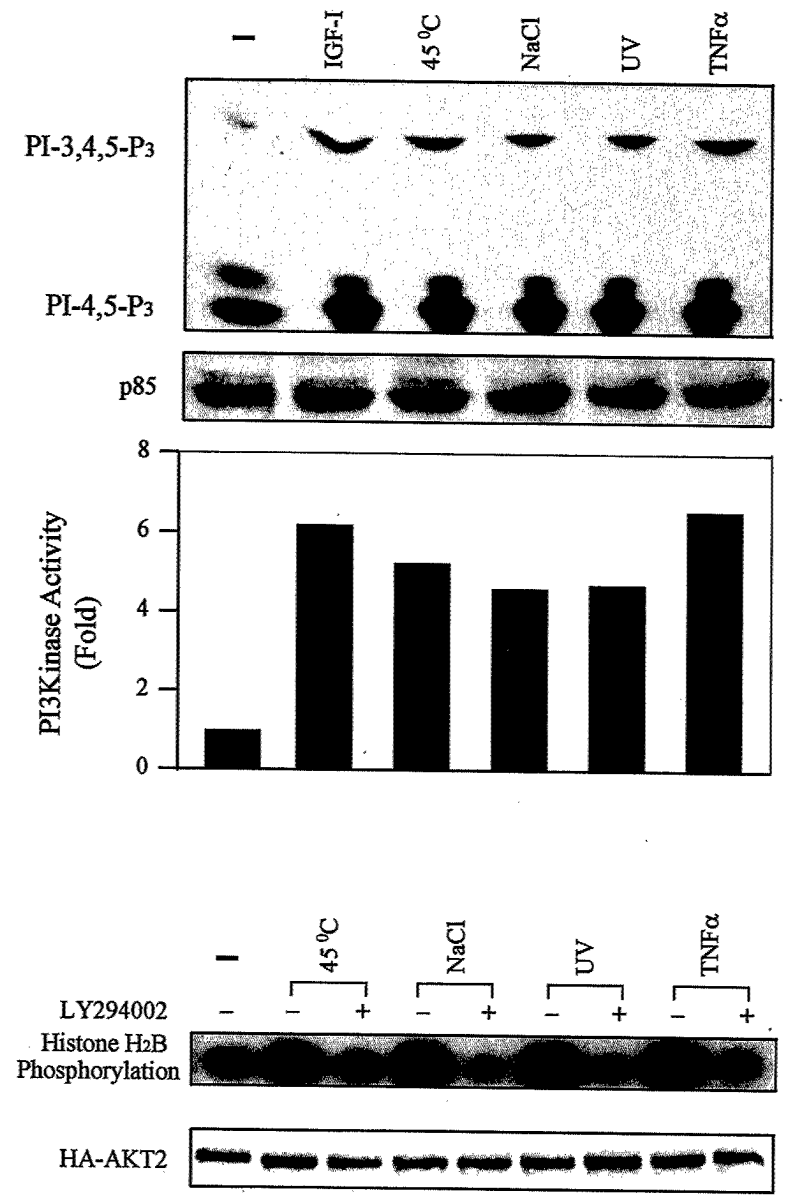


FIG. 2. **Activation of AKT2 by cellular stress and TNFα is PI3K-dependent.** *A*, *in vitro* PI3K assay. HA-AKT2-transfected HEK293 cells were exposed to the indicated stimuli. *Upper panel*, PI3K immunoprecipitates were prepared with anti-pan-p85 antibody and assayed for PI3K activity. The *middle panel* shows the p85 protein level using anti-p85 antibody, and the *bottom panel* represents the relative PI3K activity quantified by phosphorimaging. *B*, HA-AKT2-transfected A2780 cells were treated with LY294002 for 30 min before exposure to indicated stimuli. HA-AKT2 immunoprecipitates were subjected to *in vitro* kinase assay. Results were confirmed by four independent experiments. *PI-3,4,5-P_3*, phosphatidylinositol 3,4,5-trisphosphate; *PI-4,5-P_3*, phosphatidylinositol 4,5-trisphosphate.

mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the antibodies described above or with the appropriate antibodies as noted in figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting Analysis System (Amersham Biosciences).

In Vitro Protein Kinase Assay—Protein kinase assays were performed as previously described (26, 27). Briefly, reactions were carried out in the presence of 10 μCi of [γ-^{32}P] ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. Histone H2B was used as exogenous substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

PI3K Assay—PI3K was immunoprecipitated from the cell lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold phosphate-buffered saline, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in the immunoprecipitates was determined by incubating the beads in reaction buffer (10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [γ-^{32}P]ATP and 10 μg L-α-phosphatidylinositol 4,5-bisphosphate (Biomol) for 20 min at 25 °C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/MeOH, and phosphorylated products were separated by thin-layer chromatography as previously described (24). The conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate was detected by autoradiography and quantitated with a PhosphorImager.

NFκB Transcriptional Activation Analysis—HEK293 cells were seeded in 60-mm dishes and transfected with 1.5 μg of NFκB reporter plasmid (pElam-luc), 0.8 μg of pSV2-β-gal, and different forms (wild type, constitutively active, or dominant-negative) of HA-AKT2 or vector alone. The total amount of DNA transfected was increased to 6 μg with empty vector DNA. After serum starvation overnight, the cells were treated with UV (40 J/m^2) or TNFα (20 ng/ml) and lysed with 400 μl/dish of reporter lysis buffer (Tropix). The cell lysates were cleared by centrifugation for 2 min at 4 °C. Luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Assay—AKT2 stably transfected A2780 cells were seeded into 60-mm dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 24 h and pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. Apoptosis was determined by TUNEL using an *in situ* cell death detection kit (Roche Molecular Biochemicals). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in phosphate-

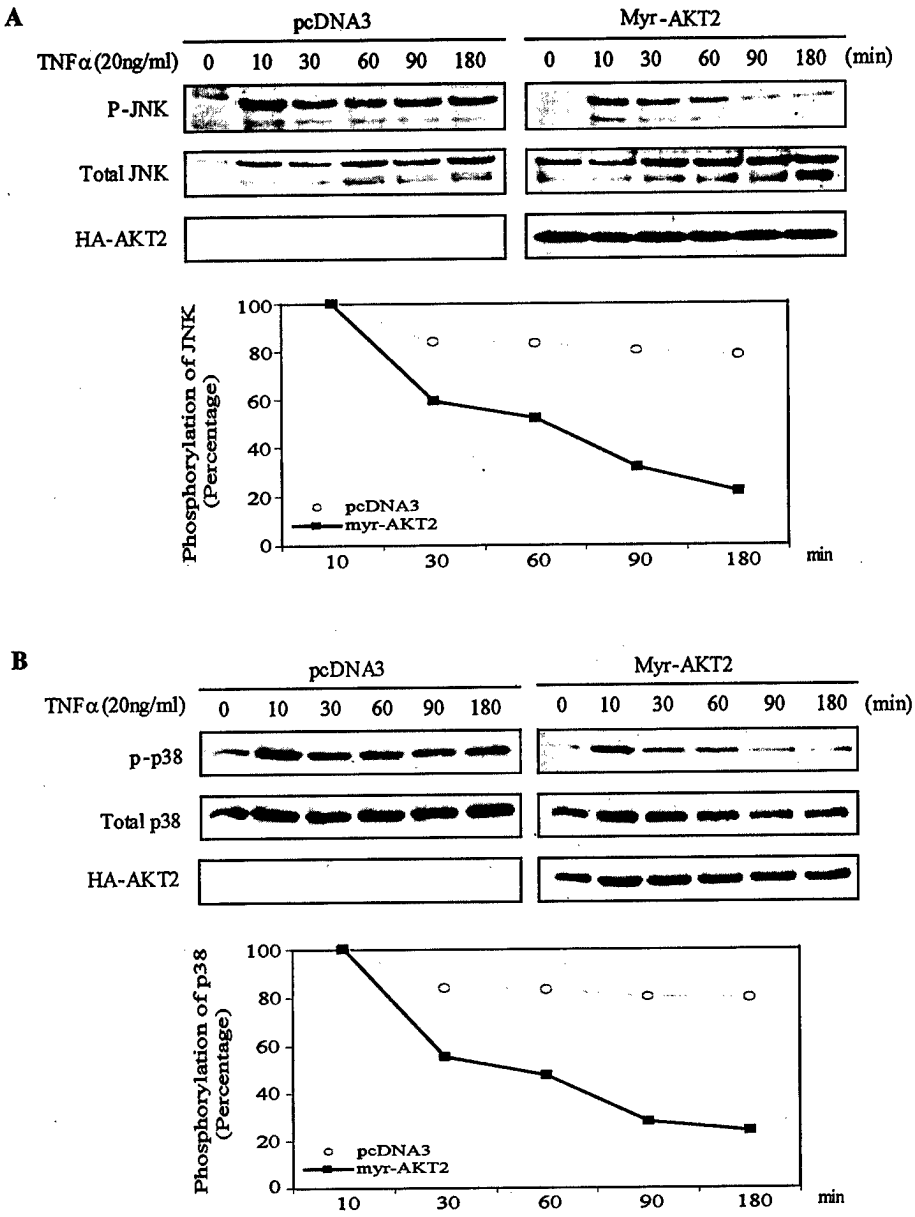


FIG. 3. **AKT2 kinase inhibits UV- and TNFα-induced JNK and p38 activation.** *A*, Western blotting analyses of HEK293 cells transfected with the indicated plasmids. Cells were lysed at indicated times after incubation with TNFα and analyzed with anti-phospho-JNK (*P-JNK, upper*), -total JNK (*middle*), and -HA (*lower*) antibodies. The immunoblotting analyses were repeated three times. *B*, the procedures are the same as *A*, except the membranes were probed with anti-phospho-p38 (*upper*), -total p38 (*middle*), and -HA (*lower*). Graphical presentations show the normalized density of phosphorylated JNK (*bottom* of *panel A*) and p38 (*bottom* of *panel B*), decaying from 100%.

buffered saline (pH 7.4)). Slides were rinsed with phosphate-buffered saline and incubated in permeabilization solution followed by TUNEL reaction mixture for 60 min at 37 °C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37 °C and then detected with alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA) for 10 min at 25 °C. After an additional rinse, the slides were mounted and analyzed under a light microscope. These experiments were performed in triplicate.

RESULTS

AKT2 Is Activated by UV Irradiation, Heat Shock, Hyperosmolarity, and TNFα—Previous studies showed that stress activates AKT1 and AKT3 but not AKT2 in fibroblasts (19). It has also been shown that TNFα receptor mediates UV- and heat shock-induced stress signaling (1–3). In agreement with these studies, we found that exposure of NIH 3T3 fibroblasts to UV-C, heat, or hyperosmotic conditions did not result in AKT2 activation (data not shown). It is possible, however, that stress might activate AKT2 in epithelial cells due to the fact of frequent alterations of AKT2, but not AKT1 and AKT3, in human epithelial tumors (7, 24, 27). For this reason we examined the effects of stress on AKT2 activation in two ovarian epithelial cancer cell lines, A2780 cells, which were transiently transfected with HA-AKT2, and OVCAR3 cells, which express high levels of endogenous AKT2 (7). The cells were exposed to UV-C, heat shock (45 °C), 0.4 M NaCl, or 20 ng/ml TNFα. IGF1-stimulated cells were used as controls. As assessed by *in vitro* kinase and Western blot analyses of AKT2 immunoprecipitates, all the stimuli substantially increased AKT2 activity in both A2780 and OVCAR3 cells (Figs. 1, *A* and *B*). The levels of AKT2 activity induced by these agents, however, were variable. AKT2 activity induced by TNFα and UV was comparable with that stimulated by IGF-1, whereas the effect of heat shock and hyperosmolarity (NaCl) on AKT2 activity was relatively smaller (Fig. 1). Nevertheless, these findings suggest that stresses activate AKT2 in a cell type-specific manner.

Stress Simulates PI3K That Mediates AKT2 Activation—To show that stress does indeed activate PI3K in epithelial cells, A2780 or HEK293 cells were exposed to UV irradiation, heat shock, and 0.4 M NaCl or TNFα, and cell lysates were immunoprecipitated with antibody to pan-p85, a regulatory subunit of PI3K. Assay of PI3K activity shows that these stress conditions as well as TNFα activated PI3K as efficiently as did IGF-1 (Fig. 2*A*). As described above, stress has been shown to activate AKT1 by both PI3K-dependent and -independent pathways

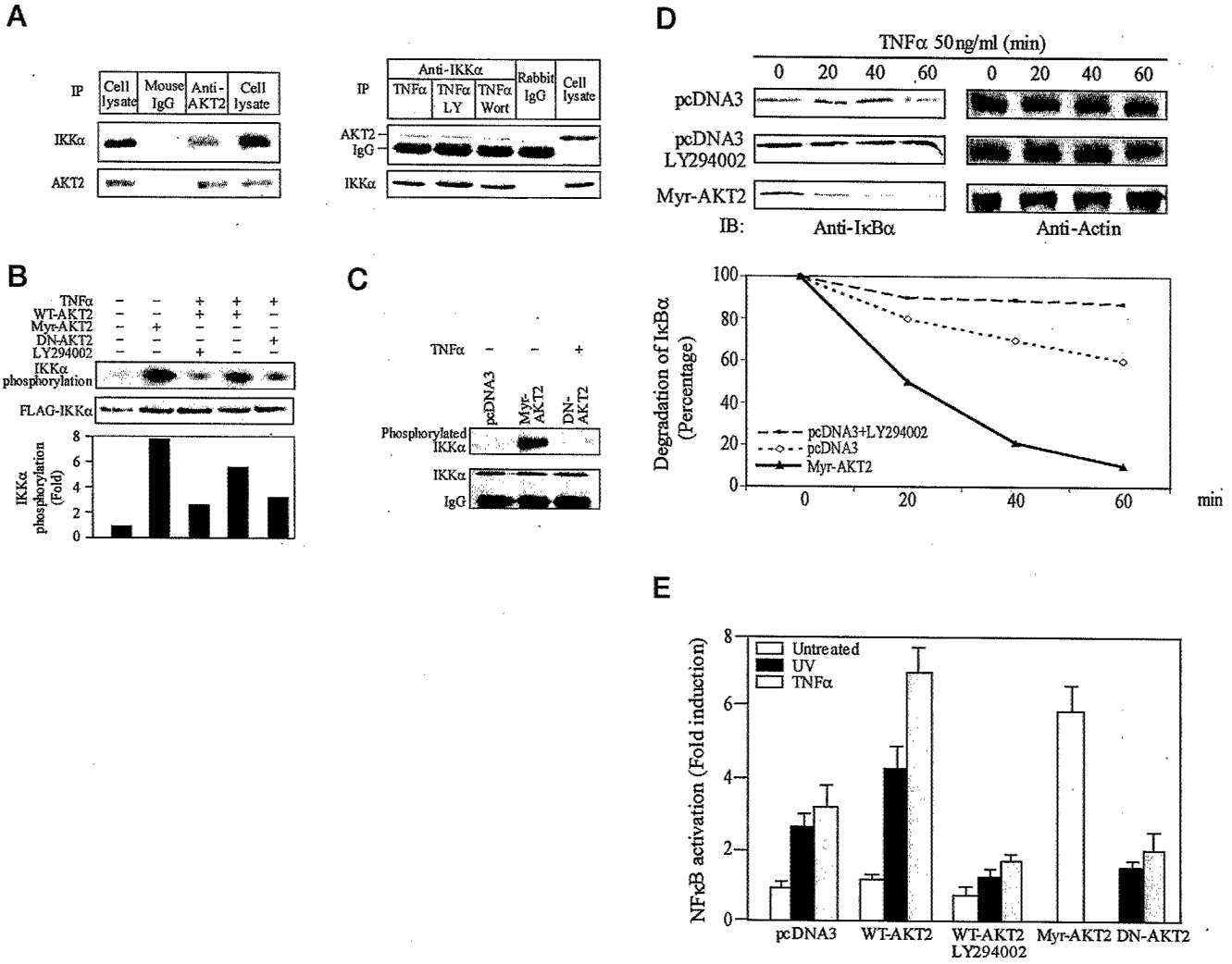


FIG. 4. **AKT2 interacts with and phosphorylates IKKα, leading to IκBα degradation and NFκB activation.** *A*, *left panel*, Western blotting analyses. HEK293 cell lysates were immunoprecipitated (*IP*) with anti-AKT2 or IgG (control) and detected with anti-IKKα (*top*) or anti-AKT2 (*bottom*) antibody. *Right panel*, HEK293 cells were treated with LY294002 (*LY*) or wortmannin for 30 min followed by TNFα for 20 min. Immunoprecipitates were prepared with anti-IKKα antibody or IgG and immunoblotted with antibody to AKT2 (*top*) or IKKα (*bottom*). *B*, *in vitro* kinase assay analyses of immunoprecipitates prepared from A2780 cells transfected with indicated plasmids using immunopurified FLAG-IKKα as substrates (*upper*). Expression of FLAG-IKKα was confirmed by immunoblotting analysis with anti-FLAG antibody (*middle*). The *bottom panel* shows the relative phosphorylation levels of IKKα by AKT2. *C*, *in vivo* labeling of IKKα from COS7 cells transfected with indicated DNA constructs treated with or without TNFα and incubated with [γ-^{32}P]orthophosphate for 4 h. IKKα immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, exposed to film (*top*), and then detected with anti-IKKα antibody (*bottom*). *D*, AKT2 induces IκBα degradation. HEK293 cells were transfected with indicated plasmids and treated with cycloheximide (50 μg/ml) for 1 h before treatment with 50 ng/ml TNFα for up to 60 min. Cell lysates were immunoblotted (*IB*) with antibody to IκBα (*left panels*) or β-actin (*right panels*). Degradation of IκBα was quantified with a densitometer. The *bottom panel* shows the degradation rate of IκBα by normalizing density of IκBα bands at 0 time point as 100%. *E*, reporter assays. HEK293 cells were transfected with 2×NFκB-Luc, β-galactosidase and WT-AKT2, Myr-AKT2, or DN-AKT2 pretreated with or without LY294002 and subsequently exposed to 40 J/m^2 UV-C or 20 ng/ml TNFα. Cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. *Error bars* represent S.D. Data were obtained from triplicate experiments.

(17, 18). To assess the role of PI3K in the stress-induced activation of AKT2, A2780 cells transfected with HA-AKT2 were exposed to 25 μM LY294002, a specific PI3K inhibitor, for 30 min before stress or TNFα treatments. LY294002 effectively inhibited stress- and TNFα-induced AKT2 activation (Fig. 2*B*). These data provide direct evidences of stress-induced activation of AKT2 through a PI3K-dependent pathway in human epithelial cells.

Stress-induced AKT2 Activation Inhibits UV- and TNFα-induced JNK and p38 Activities—Previous studies demonstrated that two groups of mitogen-activated protein kinases, the JNK and p38, are activated by environmental stress and TNFα (28). Therefore, we examined the effects of stress-induced AKT2 activation on the JNK and p38 to determine whether stressed-induced AKT2 activation could target these two stress kinases. A2780 cells were transfected with constitutively active AKT2 or pcDNA3 vector alone. Thirty-six hours after transfection, cells were treated with TNFα or UV and analyzed by Western blot for JNK and p38 activation using anti-phospho-JNK and anti-phospho-p38 antibodies. Both JNK and p38 were activated by TNFα and UV irradiation. The maximal activation was observed at 10 min of stimulation. Expression of constitutively active AKT2, however, exhibited inhibitory effects on the activation of JNK and p38 that was induced by TNFα and UV irradiation. Notably, the activation of JNK and p38 in constitutively active AKT2-transfected cells does not significantly differ from that of the cells transfected with pcDNA3 vector at 10 min of TNFα treatment. However, the phosphorylation levels of JNK and p38 in the cells expressing constitutively active AKT2 declined much more than that of

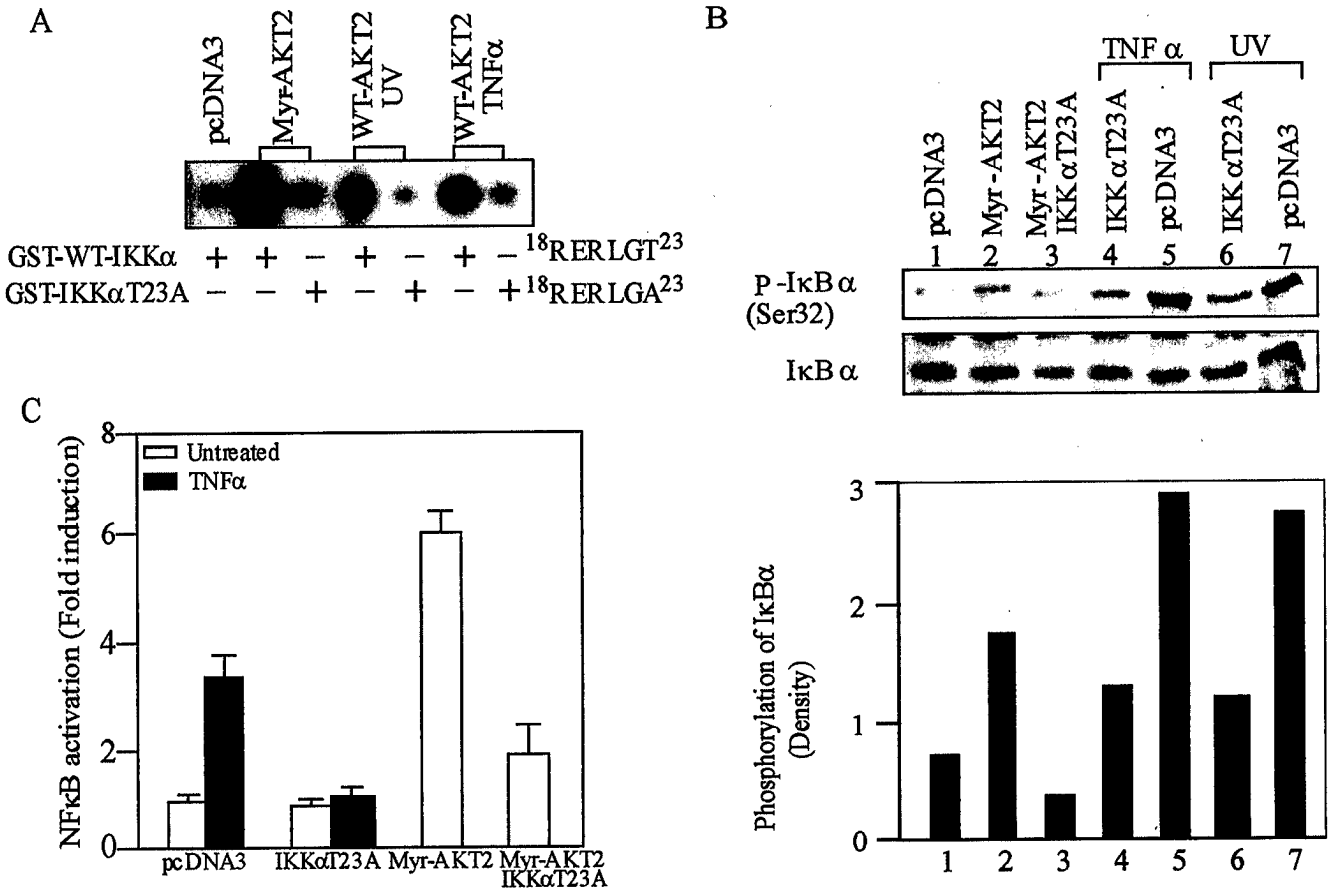


FIG. 5. **AKT2-phosphorylated IKKα at threonine 23 is required for stress-induced NFκB.** *A*, AKT2 phosphorylation of IKKα at threonine 23. *In vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transfected with the indicated plasmids and treated with or without UV or TNFα. GST-fused wild type IKKα (18**RERLGT**23) or mutant IKKα (18**RERLGA**23) was used as the substrate. *B*, AKT2 induces IκBα phosphorylation (*P-*). HEK293 cells were transfected with the indicated expression constructs. Thirty-six hours after transfection, cells were treated with 20 ng/ml TNFα for 30 min or irradiated with 40 J/m^2 UV followed by a 30-min incubation. Cell lysates were immunoblotted with anti-phospho-IκBα (*upper*) or anti-IκBα (*middle*) antibody. The band density of the phospho-IκBα was quantified (*bottom*). *C*, luciferase reporter assay. HEK293 cells were transfected with the indicated plasmids. After treatment with or without 20 ng/ml TNFα for 12 h, cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. Results were obtained from three independent experiments.

pcDNA3-transfected cells after 30 min of stimulation (Fig. 3 and data not shown). We therefore conclude that the activation of AKT2 does not activate but rather inhibits TNFα- and UV-induced JNK and p38 activities.

AKT2 Interacts With and Phosphorylates IKKα, but Not NIK, Leading to IκBα Degradation and NFκB Activation—The capacity of both cellular stress and TNFα to activate the NFκB pathway is well documented (29). Previous studies also show that AKT1 induces activation of the NFκB by interaction with IKKα (13, 14). However, to date there are no reports addressing the potential role of AKT2 in the activation of the NFκB pathway. To determine whether AKT2 associates with IKKα, HEK293 cells were treated with or without TNFα, immunoprecipitated with anti-AKT2, and immunoblotted with anti-IKKα antibody or vice versa. In both instances, the association of AKT2 with IKKα was observed (Fig. 4*A*). Additional studies showed that AKT2-IKKα interaction was unaffected by treatment of cells with PI3K inhibitor, wortmannin, or LY294002 (Fig. 4*A*). These findings indicate that AKT2 constitutively associates with IKKα. In addition, we have identified putative AKT2 phosphorylation sites in the IKKα (18**RERLGT**23) and in NFκB-inducing kinase (NIK, 366**RSREPS**371) (bold residue letters represent Akt consensus sequence). To determine whether IKKα and/or NIK are phosphorylated by AKT2, A2780 cells were transfected with different forms of AKT2 and treated with LY294002 and TNFα. *In vitro* AKT2 kinase assays were performed using FLAG-IKKα or HA-NIK, purified from the transfected COS7 cells, as substrate. Repeated experiments show that TNFα-induced AKT2 and constitutively active AKT2 phosphorylated IKKα (Fig. 4*B*) but not NIK (data not shown). Phosphorylation of IKKα induced by TNFα was largely attenuated by PI3K inhibitor LY294002. Quantification analyses revealed that approximate 70% of TNFα-induced IKKα phosphorylation was inhibited by pretreatment with LY294002 (Fig. 4*B*). Furthermore, we assessed AKT2 to determine if it phosphorylates IKKα *in vivo*. COS7 cells were transfected with FLAG-IKKα together with either constitutively active or dominant-negative AKT2 or vector alone and labeled with [γ-^{32}P]orthophosphate. IKKα immunoprecipitates prepared using anti-FLAG antibody were separated by SDS-PAGE and transferred to nitrocellulose. The phospho-IKKα was detected by autoradiography. As shown in Fig. 4*C*, IKKα was highly phosphorylated in cells expressing constitutively active AKT2 but not in the cells transfected with pcDNA3 and dominant-negative AKT2. Collectively, these data indicate that IKKα is an AKT2 physiological substrate.

Activation of NFκB requires its dissociation from its cytosolic inhibitor, IκB, a process dependent on the phosphorylation and consequent degradation of IκB by IKK. Thus, we next examined AKT2 to determine if it induces IκB degradation. Immunoblotting analyses revealed that constitutively active AKT2 significantly promoted IκBα degradation (Fig. 4*D*). To assess the involvement of AKT2 in NFκB activation, HEK293 cells were co-transfected with a NFκB-luciferase reporter and either

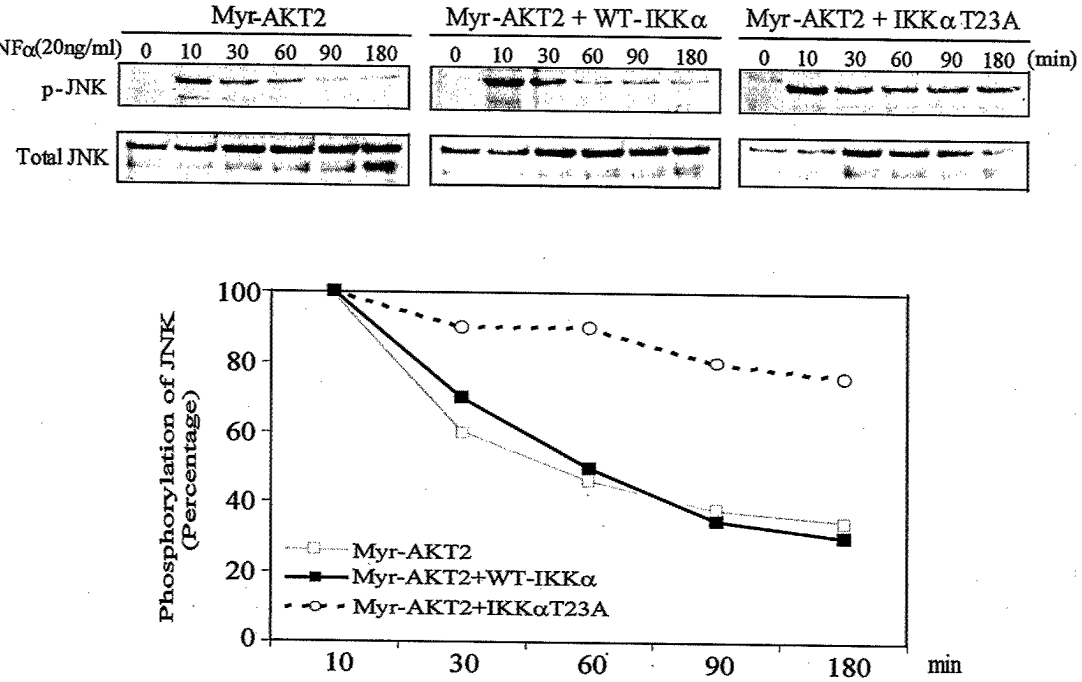


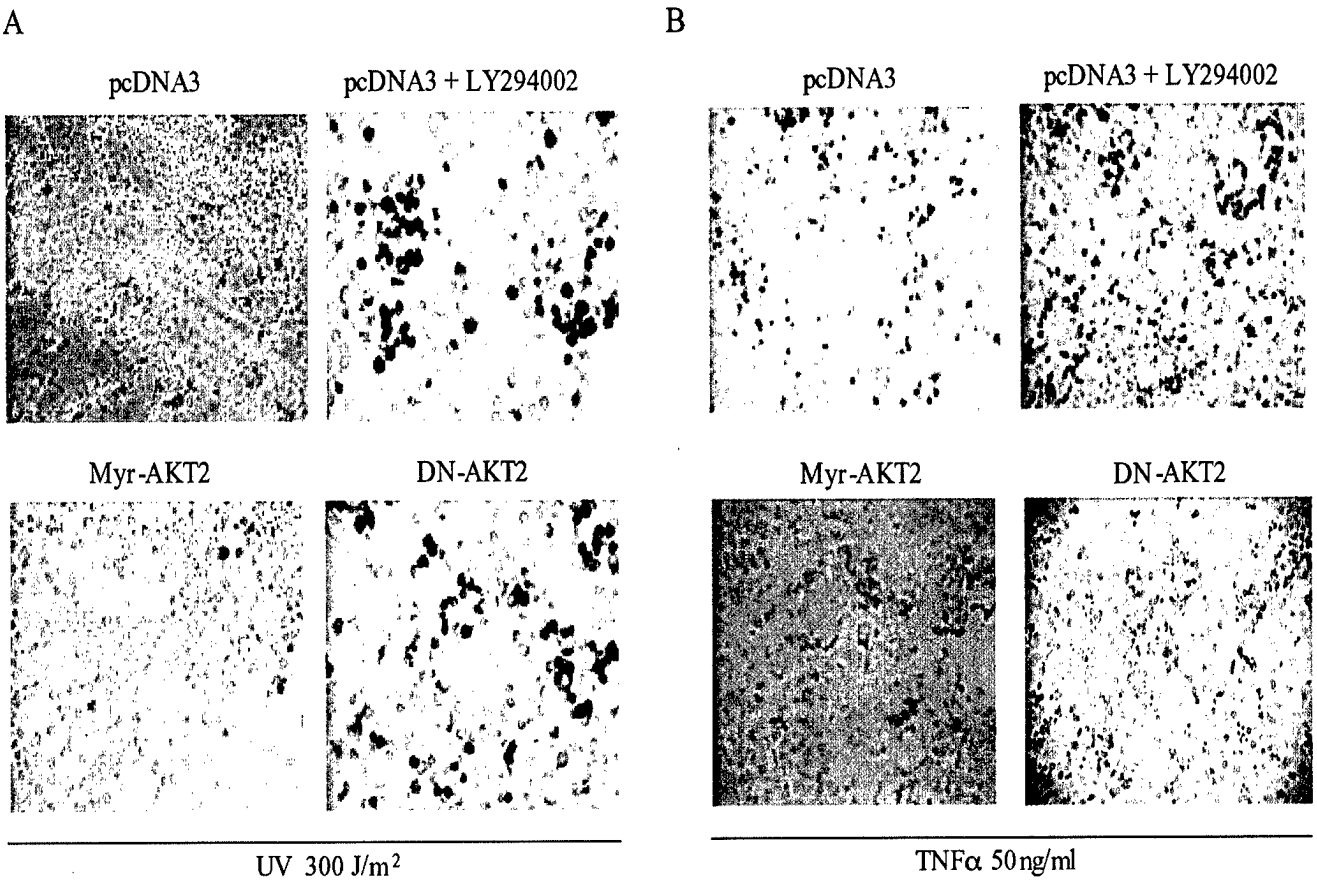
FIG. 6. **AKT2 phosphorylation of IKKα is required for inhibition of TNFα-induced JNK activity.** Immunoblotting analyses of HEK293 cells transfected with indicated expression constructs and treated with TNFα (20 ng/ml). The blots were probed with anti-phospho-JNK (*p-JNK; upper*) and -total JNK (*middle*) antibodies. Results represent one of three independent experiments. The *bottom panel* shows the quantification of phosphorylated JNK at the indicated time points.

vector alone, wild type, or constitutively active or dominant negative AKT2 treated with or without LY294002 before UV or TNFα stimulation. As shown in Fig. 4*E*, ectopic expression of wild-type AKT2 significantly enhanced UV- and TNFα-induced NFκB activity, which was abolished by treatment of cells with LY294002 or dominant negative AKT2. Constitutively active AKT2 alone was able to induce NFκB activity to a level comparable with UV- or TNFα-treated cells transfected with wild-type AKT2. These data show that PI3K/AKT2 mediates both stress- and TNFα-activated NFκB pathway.

To determine AKT2 phosphorylation site of IKKα, GST fusion proteins containing either wild type IKKα (^{18}**RERLGT**^{23}; termed GST-WT-IKKα) or mutant IKKα (^{18}**RERLGA**^{23}; termed GST-IKKαT23A) were prepared and used as substrates in *in vitro* AKT2 kinase assays. As seen in Fig. 5*A*, UV- and TNFα-activated AKT2 as well as constitutively active AKT2 phosphorylated GST-WT-IKKα but not GST-IKKαT23A. We next assessed the capacity of AKT2-induced IKKα to phosphorylate IκBα. Constitutively active AKT2 was expressed in HEK293 cells, and cell lysates were immunoblotted with an antibody that specifically recognizes phosphorylated IκBα at Ser^{32}. The results of these experiments show that constitutively active AKT2 increased IκBα phosphorylation ~2-fold and that this increase was abolished by cotransfection of pcDNA3-IKKαT23A. Expression of IKKαT23A also blocked IκBα phosphorylation induced by TNFα or UV (Fig. 5*B*). Additional luciferase reporter experiments demonstrated that expression of IKKαT23A inhibited the TNFα- or constitutively active AKT2-induced NFκB activation (Fig. 5*C*). These data indicate that phosphorylation of IKKα at Thr^{23} is required for AKT2-mediated NFκB activation.

IKKα Phosphorylation by AKT2 Is Required for Inhibition of JNK but Not p38 Activation—Recent studies showed that NFκB exerts its cell survival function by inhibition of JNK activation in response to extracellular stress (30, 31). However, it is currently unknown whether AKT-induced NFκB activation results in inhibition of JNK. Therefore, we next attempted to determine if AKT2-activated IKKα is required for AKT2 inhibition of JNK and p38 activities induced by stress and TNFα. The activation of JNK and p38 was examined in HEK293 cells transfected with IKKα or IKKαT23A together with or without constitutively active AKT2. Western blotting analyses with phospho-JNK and -p38 antibodies revealed that wild type IKKα did not significantly enhance AKT2 inhibition of JNK (Fig. 6). However, expression of IKKαT23A abrogated the effects of constitutively active AKT2 on inhibition of JNK (Fig. 6). Similar to the results shown in Fig. 3, TNFα-induced JNK activation reached the maximal level at 10 min of stimulation, which was neither significantly inhibited by constitutively active AKT2 nor affected by expression of IKKαT23A (Fig. 6). Therefore, these data indicate that inhibition of JNK activation by AKT2/NFκB could be via a mechanism of induction of dephosphorylation of JNK by the AKT2/IKKα/NFκB cascade.

AKT2 Activation Inhibits Stress-induced Apoptosis—It is documented that various stresses and TNFα are capable of inducing apoptosis in different cell types through activation of JNK and p38 pathways (29). Because PI3K/AKT is essential for cell survival and activated AKT2 inhibits JNK/p38 and induces NFκB pathway, we investigated the role of PI3K/AKT2 in stress- and TNFα-induced programmed cell death. AKT2 stably transfected A2780 cells were pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. As determined by the TUNEL assay, inhibition of PI3K activity dramatically increased the percentage of cells undergoing apoptosis in response to UV or TNFα (Fig. 7). Moreover, inhibition of AKT2 activity by expression of dominant-negative AKT2 increased the percentage of apoptotic cells in the UV- and TNFα-treated populations by ~2-fold. On the other hand, cells expressing constitutively active AKT2 were resistant to UV- and TNFα-induced apoptosis. These data show that the PI3K/AKT2 pathway plays a key role in protecting cells from apoptosis induced by extracellular stress or TNFα.



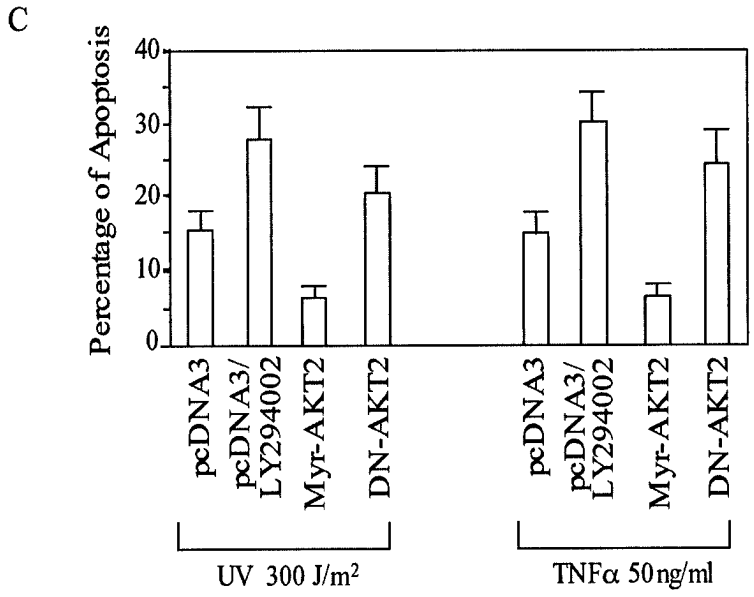


FIG. 7. **AKT2 activation inhibits stress-induced apoptosis.** A2780 cells stably transfected with constitutively active AKT2, DN-AKT2, or vector alone were pretreated with or without 25 μM LY294002 for 2 h before exposure to UV-C (300 J/m²) (*A*) or TNFα (50 ng/ml) for 24 h (*B*). Apoptosis was assessed by TUNEL assay. *C*, quantitation of data shown in *A* and *B* were derived from triplicate experiments. *Error bars* represent S.D.

DISCUSSION

In this report, we have provided evidence that AKT2 is activated by extracellular stress and TNFα through a PI3K-dependent pathway in human epithelial cells. Most importantly, the activation of AKT2 inhibits stress- and TNFα-induced JNK and p38 activities and activates the NFκB cascade, leading to protection of cells from stress- and TNFα-induced apoptosis.

Previous studies show that stress activates cell membrane

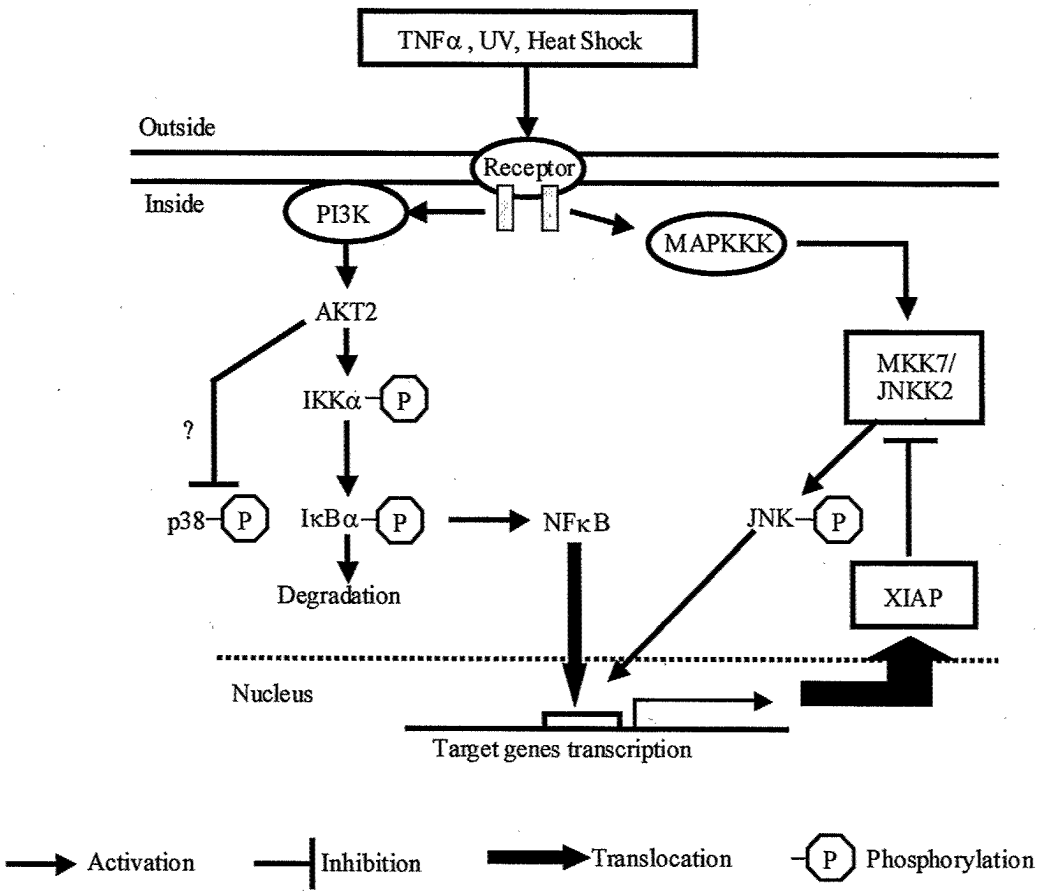


FIG. 8. **Schematic illustration of negative regulation of JNK by AKT2/NFκB.**

receptors, including those for epidermal growth factor, platelet-derived growth factor, and IGF. As a result, receptors associate with numerous proteins that activate downstream signaling molecules (1–3). One such protein is PI3K, which has been implicated in the regulation of nearly all stress signaling pathways (1). Because the AKTs are major downstream targets of PI3K, their role in the stress response has been recently investigated. In Swiss 3T3 cells, both oxidative stress and heat shock were shown to induce a marked activation of AKT1 and AKT3 but not AKT2 (19). AKT1 activation by hyperosmotic stress in COS7 and NIH 3T3 cells has also been demonstrated (17). In this study, we show that AKT2 is activated by different stress conditions including UV irradiation, hyperosmolarity, and heat shock as well as by TNFα in several human epithelial cell lines.

Three isoforms of AKT display high sequence homology and share similar upstream regulators and downstream targets as identified so far. However, there are clear differences between them in terms of biological and physiological function. In addition to the more prominent role of AKT2 in human malignancy and transformation (7, 32), the expression patterns of AKT1, AKT2, and AKT3 in normal adult tissues as well as during development are quite different (4, 8, 33). Recent studies suggest that AKT1, AKT2, and AKT3 may interact with different proteins and, thus, may play different roles in signal transduction. For instance, the Tcl1 oncoprotein preferentially binds to and activates AKT1 but not AKT2 (34). Gene knockout studies revealed that AKT1-deficient mice display defects in both fetal and postnatal growth but, unlike $AKT2^{-/-}$ mice, do not exhibit a type II diabetic phenotype; these differences suggest that the functions of AKT1 and AKT2 are non-redundant with respect to organismic growth and insulin-regulated glucose metabolism (21–23). It has been also shown that AKT2 but not AKT1 plays a specific role in muscle differentiation (35).[2] In this study, we demonstrated that AKT2 is activated by a variety of stress conditions in human epithelial cells but not in fibroblasts, suggesting that activation of different isoforms of AKT is cell type-specific in response to extracellular stress.

It is controversial whether stress-induced AKT1 activation is mediated by the PI3K pathway (17–19). Two previous reports showed that PI3K inhibitors did not block heat shock- or H_2O_2-induced activation of AKT1 and, thus, suggested that stress (unlike growth factors) activates AKT1 in a PI3K-indpendent manner (17, 18). However, the opposite results were observed by other groups (19, 20). Konishi *et al.* also provide evidence of AKT1 activation by H_2O_2 and heat shock through both PI3K-dependent and -independent pathways (18). We previously demonstrated that activation of AKT2 by growth factors required PI3K activity, whereas both PI3K-dependent and -independent pathways contributed to AKT2 activation by Ras (26). In this report, we show that PI3K inhibitors completely block AKT2 activation induced by UV-C, heat shock, and hyperosmolarity, indicating that stress activates AKT2 via the PI3K pathway.

JNK and p38 are stress mitogen-activated protein kinases that are activated by cytokines and a variety of cellular stresses (28). Like the classical mitogen-activated protein kinase kinase (MEK), direct activators for JNK and p38 have been identified. JNK is activated by phosphorylation of tyrosine and threonine by the dual specificity kinases, MKK4/SEK1 and MKK7. Similarly, p38 is activated by MKK3 and MKK6. However, biochemical studies have documented the existence of other JNK

[2] S. Kaneko, S. V. Nicosia, Z. Wu, T. Nobori, and J. Q. Cheng, submitted for publication.

and p38 activators or inhibitors in cells stimulated by a variety of cellular stresses (28). Although previous reports showed that AKT, JNK, and p38 are downstream targets of PI3K and represent parallel pathways in response to stress (17–20, 37, 38), the data presented in this study indicate that stress- and TNFα-induced activation of AKT2 inhibits the JNK and p38 activities, suggesting that AKT2 cross-talks with JNK and p38 stress pathways.

NFκB is another critical stress response pathway (29). Activation of NFκB is achieved through the signal-induced proteolytic degradation of IκB, which is associated with and inhibits the activity of NFκB in the cytoplasm. The critical event that initiates IκB degradation is the stimulus-dependent activation of the IκB kinases IKKα and IKKβ, which phosphorylate IκB at specific N-terminal serine residues (Ser^{32} and Ser^{36} for IκBα; Ser^{19} and Ser^{23} for IκBβ). Phosphorylated IκB is then selectively ubiquitinated by an E3 ubiquitin ligase and degraded by the 26 S proteasome, thereby releasing NFκB for translocation to the nucleus where it initiates the transcription of target genes (29). Moreover, two mitogen-activated protein kinase kinase kinase (MAPKKK) members, NIK and MEKK1, have been reported to enhance the activity of the IKKs and consequently trigger the phosphorylation and destruction of the IκBs and induce the activation of the NFκB pathway (29). Recent studies also showed that AKT1 induces the NFκB cascade through activation of IKK and degradation of IκB (13, 14). In this report, we show that AKT2 physically binds to and phosphorylates IKKα but not NIK even though NIK contains an AKT2 phosphorylation consensus sequence. When activated by stress or TNFα, AKT2 degrades IκB and activates NFκB-mediated transcription, indicating that stress-activated AKT2 targets the NFκB pathway.

Importantly, we have provided evidence that activation of AKT2 induced by stress and TNFα inhibits JNK activity through activation of the NFκB pathway to protect cells from apoptosis in response to these stimuli. Previous studies showed that the AKT2 pathway is important for cell survival and malignant transformation (7, 24, 32). The data presented here show that cells expressing constitutively active AKT2 are resistant to stress- and TNFα-induced apoptosis and that dominant-negative AKT2 and LY294002 sensitize cells to stress- and TNFα-induced programmed cell death. These findings indicate that stress-induced AKT2 activation promotes cell survival. Among the stress-activated kinases are JNK; recent studies demonstrated that activation of JNK and p38 plays an important role in triggering apoptosis in response to extracellular stress and TNFα (36, 39–41), whereas activation of NFκB protects cells from programmed cell death (29). Although a number of downstream targets of AKT2 have been identified, our data indicate that AKT2-inhibited JNK and p38 activities and AKT2-induced NFκB activation could play, at least in part, an important role in the AKT2 pathway that protects cells from stress- and TNFα-induced apoptosis. Recent reports demonstrate that NFκB-up-regulated Gadd45β and Xiap inhibited JNK activation and abrogated TNFα-induced programmed cell death (30, 31). Our cDNA microarray experiments showed that constitutively active AKT2 induces *Xiap*.[3] Thus, AKT2 inhibition of JNK activity could be due to up-regulation of Xiap by NFκB pathway (Fig. 8). Further studies are required to characterize the mechanism of inhibition of p38 stress pathway by AKT2 and involvement of Xiap in AKT2/NFκB inhibition of the JNK activation.

[3] M. Sun and J. Q. Cheng, unpublished data.

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Phosphatidylinositol 3-Kinase/Akt Pathway Regulates Tuberous Sclerosis Tumor Suppressor Complex by Phosphorylation of Tuberin*

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Normal cellular functions of hamartin and tuberin, encoded by the *TSC1* and *TSC2* tumor suppressor genes, are closely related to their direct interactions. However, the regulation of the hamartin-tuberin complex in the context of the physiologic role as tumor suppressor genes has not been documented. Here we show that insulin or insulin growth factor (IGF) 1 stimulates phosphorylation of tuberin, which is inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 but not by the mitogen-activated protein kinase inhibitor PD98059. Expression of constitutively active PI3K or active Akt, including Akt1 and Akt2, induces tuberin phosphorylation. We further demonstrate that Akt/PKB associates with hamartin-tuberin complexes, promoting phosphorylation of tuberin and increased degradation of hamartin-tuberin complexes. The ability to form complexes, however, is not blocked. Akt also inhibits tuberin-mediated degradation of $p27^{kip1}$, thereby promoting CDK2 activity and cellular proliferation. Our results indicate that tuberin is a direct physiological substrate of Akt and that phosphorylation of tuberin by PI3K/Akt is a major mechanism controlling hamartin-tuberin function.

Tuberous sclerosis complex (TSC)[1] is an autosomal dominant disorder and is characterized by the presence of hamartomas in many organs such as brain, skin, heart, lung, and kidney (1). It is caused by mutation of either the *TSC1* or *TSC2* tumor suppressor gene (2–5). *TSC1* encodes a protein, hamartin, containing two coiled-coil domains that have been shown to mediate binding to hamartin (6). The *TSC2* gene codes for tuberin, which contains a small region of homology to the rap1GTPase-activating protein, rap1GAP (7). These two proteins function within the same pathway(s) regulating cell cycle, cell growth, adhesion, and vesicular trafficking (4, 5). However, the regulation of hamartin and tuberin in the context of physiologic role as tumor suppressor genes has not been documented.

Among the various properties of these two proteins, the ability to interact and to form stable complex has been the most consistent finding. This led to the hypothesis that hamartin and tuberin function as a complex and that factors regulating their interaction are important in understanding physiologic roles. There is evidence to suggest that phosphorylation of tuberin may be a major mechanism of regulation of the hamartin-tuberin complex (8, 9). However, the kinases that are responsible for phosphorylation of this complex are currently unknown. Recent *Drosophila* genetic studies showed that *dTsc1* and *dTsc2* play an important role in the insulin/dPI3K/dakt signal transduction pathway by demonstrating that reduced cell size and cell proliferation caused by either mutations in *dINR* and *dakt* or by overexpression of *dPTEN* are overridden by homozygous mutants of *dTsc1* or *dTsc2*. This implies that *dTsc1* and *dTsc2* are either direct downstream targets of dakt or on a parallel pathway of the insulin cascade downstream from dakt (10–13). Akt, also known as protein kinase B (PKB), represents a subfamily of the serine/threonine protein kinase. Three isoforms of Akt have been identified including Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, all of which are activated by growth factors and insulin in a PI3K-dependent manner and are inhibited by PTEN tumor suppressor (14). Akt regulates a wide spectrum of cell functions, including cell survival, cell growth, differentiation, angiogenesis, and glucose metabolism, through phosphorylation of a number of proteins that contain the RXRXXS/T motif (14–16).

Here we show that Akt physically interacts with and phosphorylates tuberin, leading to degradation of the hamartin-tuberin complex and $p27^{kip1}$ without interfering with hamartin-tuberin complex formation. Moreover, IGF1 and insulin induce tuberin phosphorylation, which is mediated by the PI3K/Akt pathway but not by the MAPK pathway. As a result, cyclin-dependent kinase (CDK) 2 activity, DNA synthesis, and S phase of the cell cycle are elevated. We thus have identified Akt as a major tuberin kinase to negatively regulate hamartin-tuberin tumor suppressor function by inducing degradation.

EXPERIMENTAL PROCEDURES

Plasmids—The cytomegalovirus-based expression constructs encoding wild type, constitutively active, and dominant negative Akt, Myc-TSC1, and TSC2-Xpress have been described (3, 17). TSC2-7A and

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[1] The abbreviations used are: TSC, tuberous sclerosis complex; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; IGF, insulin-like growth factor; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HEK, human embryonic kidney; GST, glutathione *S*-transferase; CDK, cyclin-dependent kinase.

TSC2-7D mutant constructs were created using a QuikChange multiple site-directed mutagenesis kit (Stratagene). Constitutively active PI3K (p110*) was provided by Julian Downward (London, UK).

Cell Culture, Transfection, and Flow Cytometry—Human embryonic kidney (HEK) 293 and HeLa cells were obtained from the American Type Culture Collection. EEF4 (TSC2-positive) and EEF8 (TSC2-negative) cells were derived from Eker rat embryos homozygous for the wild type and the Eker-mutant TSC2 gene, respectively (8). All cells were grown either in Dulbecco's modified Eagle's medium or in RPMI 1640 medium, both supplemented with 10% calf serum and antibiotics. Cell transfections were performed using LipofectAMINE Plus. For cytofluorometric analyses, cells were harvested by trypsinization, fixed, and analyzed on a FACScan.

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assay—For immunoprecipitation, lysates were incubated with the appropriate antibody (as noted in the figure legends) in the presence of protein A-protein G (2:1)-agarose beads. The beads were washed with lysis buffer. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Detection of antigen-bound antibody was carried out with the ECL System. Protein kinase assays were performed as described previously (18).

Pulse-chase Experiments—Prior to radioactive labeling normal culture medium was removed, and cells were washed twice with phosphate-buffered saline and refed with minimum Eagle's medium lacking methionine but supplemented with 10% dialyzed fetal bovine serum and 300 μCi of Tran^{35}S-label per plate. After 60 min of labeling, cells were lysed and immunoprecipitated with anti-TSC1, -TSC2, or -p27 antibody. The immunoprecipitates were separated by SDS-PAGE gel. Gels were dried and autoradiographed. Quantification of bands was performed with a PhosphorImager.

In Vivo [^{32}P]orthophosphate Cell Labeling—COS7 cells were transfected with pcDNA3-TSC2 together with or without constitutively active Akt and labeled with [^{32}P]orthophosphate (0.5 mCi/ml) in minimum Eagle's medium without phosphate for 4 h. Tuberin was immunoprecipitated with anti-TSC2 antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated tuberin was detected by autoradiography.

RESULTS

Tuberin Is a Physiological Substrate of Akt—Recent studies demonstrated that tuberin is phosphorylated at serine and tyrosine residues in response to serum, phosphatase inhibitors, and anisomycin and that the phosphorylated tuberin regulates its interaction with hamartin (8, 9). However, the kinases that are responsible for phosphorylation of tuberin are currently unknown. Because tuberin contains seven Akt phosphorylation consensus sites that are very conserved between human, rat, and mouse as well as four that are also found in *Drosophila* (Fig. 1*a*), we examined the possibility of Akt phosphorylation of tuberin. *In vivo* [^{32}P]orthophosphate cell-labeling experiments revealed that constitutively active Akt and IGF1-induced Akt significantly phosphorylate tuberin (Fig. 1*b*). To explore which sites on tuberin are potentially phosphorylated by Akt, *in vitro* kinase assay was carried out using wild type and mutant (converting S/T to alanine) GST fusion proteins for each of seven Akt putative phosphorylation sites as substrate. As shown in Fig. 1*c*, Akt can highly phosphorylate fusion proteins containing all seven serine and threonine sites of tuberin but not their mutants. We therefore conclude that tuberin is a physiological substrate of Akt.

The PI3K/Akt Pathway, but Not the MAPK Pathway, Mediates Insulin, IGF1, and Serum-induced Tuberin Phosphorylation—Because genetic studies of the *dTsc complex* in *Drosophila* have demonstrated that *dTsc1/dTsc2* antagonize insulin signaling in cell growth (10–13), we next examined whether insulin and IGF1 induce hamartin-tuberin phosphorylation and whether Akt mediates this action. Western blotting analyses showed that tuberin, but not hamartin, was phosphorylated upon insulin, IGF1, or serum stimulation in HeLa cells as demonstrated by gel mobility shift (Fig. 1*d*). The phosphorylation was abrogated by treatment with phosphatase PP2A or PI3K inhibitors, LY294002, and wortmannin, but not by MAPK

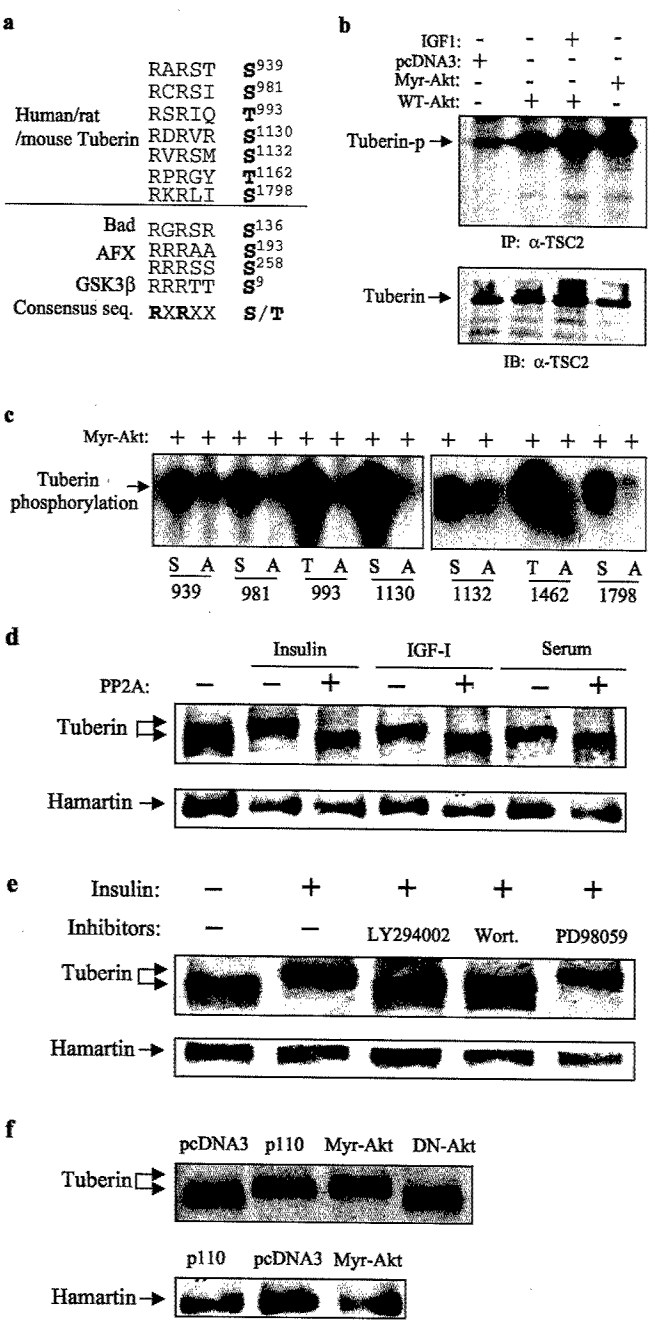

FIG. 1. **Akt phosphorylates tuberin *in vitro* and *in vivo* and mediates insulin- and IGF1-induced tuberin phosphorylation.** *a*, comparison of the putative Akt phosphorylation sites in tuberin with the sequences of phosphorylation sites of known Akt substrates. The phosphorylated residues are labeled by number, and a consensus sequence is denoted below. *b*, *in vivo* [^{32}P]orthophosphate labeling HeLa cells transfected with constructs immunoprecipitated with anti-TSC2 antibody are indicated at the *top*. Immunoprecipitates were separated by SDS-PAGE, transferred to membrane, exposed to the film (*upper panel*), and detected by anti-TSC2 antibody (*bottom panel*). *WT-Akt*, wild type Akt. *c*, *in vitro* kinase assay analysis of constitutively active Akt (*Myr-Akt*) immunoprecipitates using each of the GST-fused seven Akt phosphorylation sites and their alanine (*A*) mutants as substrate indicated at the bottom. *d–f*, Western blot analysis of tuberin in HeLa cells that were serum-starved overnight and stimulated with or without insulin, IGF1, or serum for 15 min. Tuberin was immunoprecipitated, treated with phosphatase PP2A, and immunoblotted with anti-tuberin antibody. Electrophoretic mobility shift of tuberin, *i.e.* the phosphorylated form of tuberin, was observed upon insulin, IGF1, or serum stimulation and was abrogated by treatment with PP2A (*d*) or PI3K inhibitors (*LY294002* and *wortmannin*) for 20 min (*e*). *f*, HeLa cells were transfected with constitutively active p110, Akt, and DN-Akt and immunoblotted with anti-TSC2 (*top*) and -TSC1 (*bottom*) antibodies.

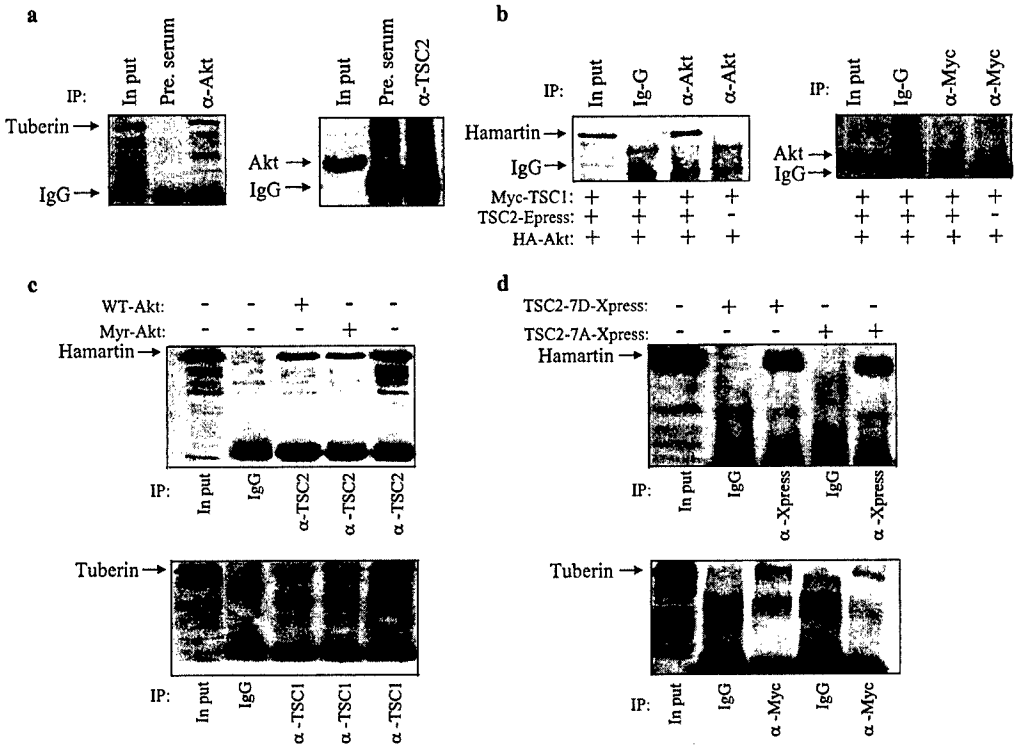

FIG. 2. **Akt interacts with tuberin but does not interfere with hamartin-tuberin complex formation.** *a*, Western blot analyses of immunoprecipitates prepared from HeLa cells with anti-Akt antibody and detected with anti-TSC2 antibody (*left*) or *vice versa* (*right*). Preimmune serum (*Pre. serum*) was used as negative control, and total cell lysate (*In put*) indicates expression of TSC2 and Akt. *b*, Akt is indirectly associated with hamartin. HEK293 cells were transfected with plasmids as indicated at the *bottom*. Immunoprecipitations were prepared with polyclonal Akt antibody and detected with monoclonal anti-Myc antibody (*left*) or *vice versa* (*right*). *c*, HeLa cells were transfected with wild type, constitutively active Akt, or pcDNA3 vector alone. Immunoprecipitations were carried out with antibodies indicated at the *bottom* of each panel and detected with anti-TSC1 (*top*) or -TSC2 (*bottom*) antibody. *d*, HEK293 cells were transfected with TSC2-7D-Xpress or TSC2-7A-Xpress, immunoprecipitated with anti-Xpress antibody, and detected with anti-Myc antibody (*top*) or *vice versa* (*bottom*).

inhibitor PD98059 (Fig. 1*e*). Furthermore, expression of constitutively active PI3K (p110*) and active Akt significantly induced the phosphorylation of tuberin (Fig. 1*f*). These data indicate that the function of TSC tumor suppressors is regulated by mitogenic growth factor IGF1 and insulin through the PI3K/Akt, but not the MAPK, pathway.

Akt Interacts with Tuberin and Hamartin—To assess whether Akt physically associates with the hamartin-tuberin complex, coimmunoprecipitation experiments were performed in HeLa cells. As seen in Fig. 2*a*, Akt directly bound to tuberin. Because hamartin and tuberin function as a complex and Akt associates with tuberin, we assumed that Akt could indirectly interact with hamartin. To test this hypothesis, HEK293 cells were transfected with Myc-TSC1, HA-Akt, and/or TSC2-Xpress. Coimmunoprecipitation experiments revealed that interaction between Akt and hamartin was only detected in the cells transfected with Myc-TSC1/TSC2-Xpress/HA-Akt but not with Myc-TSC1/Akt (Fig. 2*b*), indicating that Akt binding to hamartin is mediated by tuberin. Because tuberin binds to Akt and is phosphorylated by Akt, we conclude that tuberin is a direct downstream target of Akt.

The Hamartin-Tuberin Complex Is Not Disrupted by Akt Phosphorylation of Tuberin—Because previous studies have suggested that phosphorylation of tuberin regulates its interaction with hamartin (8, 9), we next examined whether Akt interferes with hamartin-tuberin complex formation. Coimmunoprecipitation revealed that expression of wild type and constitutively active Akt in HeLa cells did not disrupt the interaction between hamartin and tuberin (Fig. 2*c*), despite the fact that hamartin and tuberin function as a complex. Moreover, phosphomimic TSC2-7D-Xpress and nonphosphorylatable TSC2-7A-Xpress, prepared by converting seven Akt phosphorylation sites of tuberin into aspartic acid and alanine, respectively, were transfected into HeLa cells. Immunoblotting analyses of TSC2-7D-Xpress and TSC2-7A-Xpress immunoprecipitates showed that both mutant forms of tuberin still bound to hamartin (Fig. 2*d*), indicating that Akt phosphorylation of tuberin did not hamper the interaction between hamartin and tuberin.

Akt Phosphorylation of Tuberin Induces Degradation of Hamartin and Tuberin—Strikingly, we observed that expression of constitutively active Akt significantly down-regulated hamartin and tuberin in a dose-dependent manner, *i.e.* protein levels of hamartin and tuberin progressively declined when the cells were transfected with increasing amounts of constitutively active Akt. Accordingly, the protein amount of hamartin and tuberin in the complex was also decreased (Fig. 3*a*). To exclude the possibility of Akt down-regulation of hamartin and tuberin resulting from inhibition of the *TSC1* and *TSC2* gene transcription, Northern blot analyses were performed and showed that mRNA levels of *TSC1* and *TSC2* did not change in HeLa cells transfected with constitutively active Akt as compared with the cells transfected with pcDNA3 vector alone (Fig. 3*e*). Because Akt has been shown to activate rather than inhibit translation initiation through regulation of FRAP/mTOR/4E-BP (14, 15), it is unlikely that Akt regulates hamartin and tuberin at a translational level. Thus, we assumed Akt down-regulation of hamartin and tuberin occurred through protein degradation. Pulse-chase experiments revealed that expression of constitutively active Akt considerably induced hamartin and tuberin degradation (Fig. 3*b*). Moreover, the proteosome inhibitor MG132 attenuated Akt-induced hamartin and tuberin degradation (Fig. 3*c*). These data suggest that Akt down-regulation of hamartin and tuberin is mediated by a post-transla-

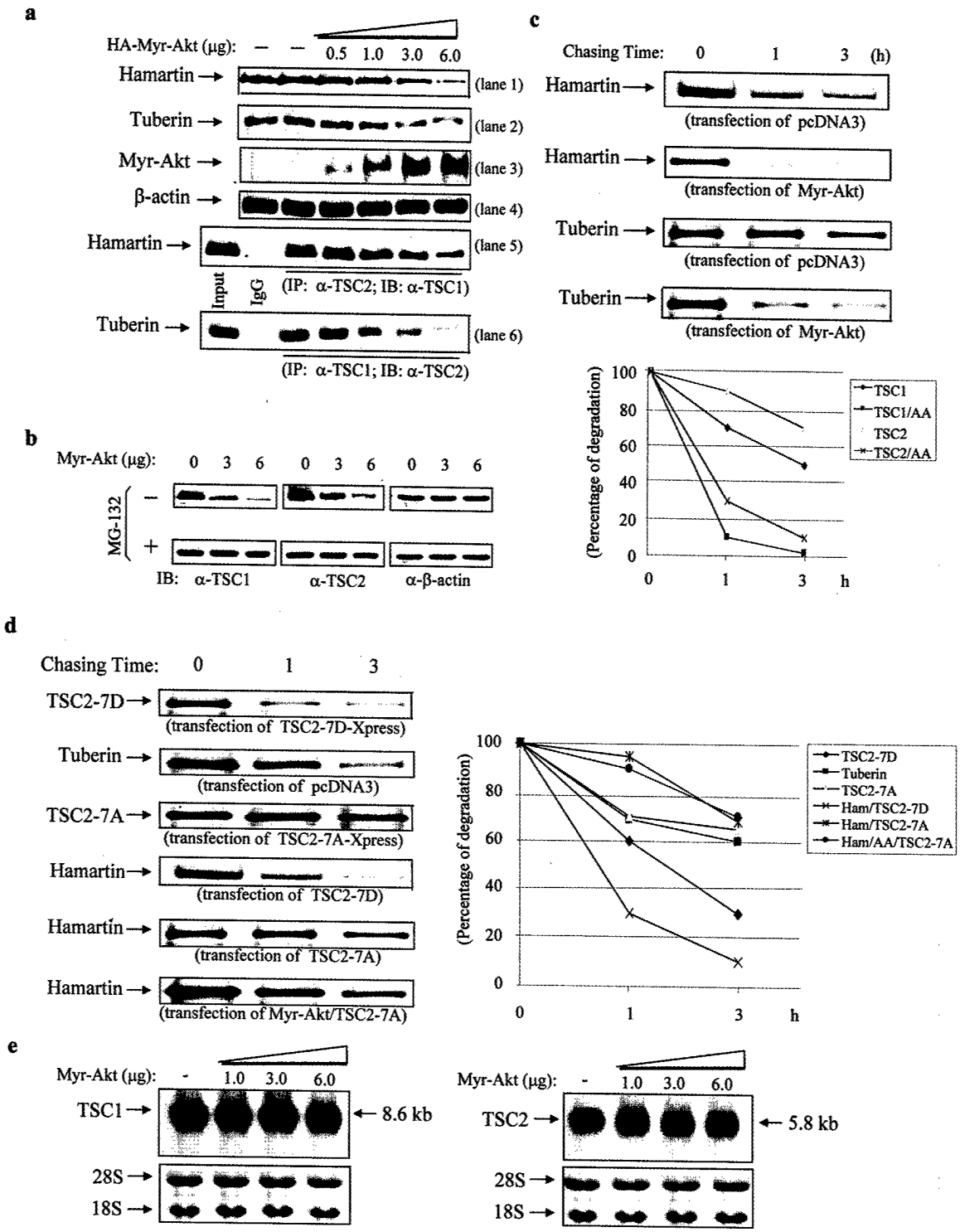

FIG. 3. **Akt phosphorylation of tuberin induces degradation of hamartin and tuberin.** *a*, HeLa cells were transfected with HA-Myr-Akt. A portion of the cell lysate was subjected to Western blot analysis using anti-TSC1 (*panel 1*), -TSC2 (*panel 2*), -HA (*panel 3*), and -β-actin (*panel 4*) antibodies. The rest were immunoprecipitated with anti-TSC2 antibody and detected with anti-TSC1 antibody (*panel 5*) or *vice versa* (*panel 6*). *b*, constitutively active Akt-transfected HeLa cells were pretreated with or without MG132 for 2 h, lysed, and subjected to immunoblotting analyses with anti-TSC1, -TSC2, and -β-actin antibodies. *c*, pulse-chase analyses of degradation of hamartin and tuberin. TSC2-positive EEF4 cells were transfected with plasmids indicated at the *bottom* of each panel, labeled with [^{35}S]methionine, chased at indicated times, and immunoprecipitated with anti-TSC1 or -TSC2 antibodies. The immunoprecipitates were separated by SDS-PAGE, exposed, and quantified. *d*, phosphomimic TSC2-7D promotes and nonphosphorylatable TSC2-7A inhibits the degradation of hamartin and tuberin. TSC2-deficient EEF8 cells were transfected with indicated expression plasmids (*bottom of each panel*) and chased at indicated times after labeling with [^{35}S]methionine. Immunoprecipitations were performed with anti-TSC1, -TSC2, or -Xpress antibodies. Graphical presentations show the normalized density of hamartin and tuberin degradation from 100%. *e*, ectopic expression of constitutively active Akt does not affect mRNA levels of TSC1 and TSC2. HeLa cells were transfected with increasing amounts of constitutively active Akt. After 48 h of transfection, total RNAs were isolated and subjected to Northern blot analyses with [^{32}P]dCTP-labeled TSC1 (*left*) or TSC2 (*right*) cDNA probe. *Bottom panels* indicate equal loadings.

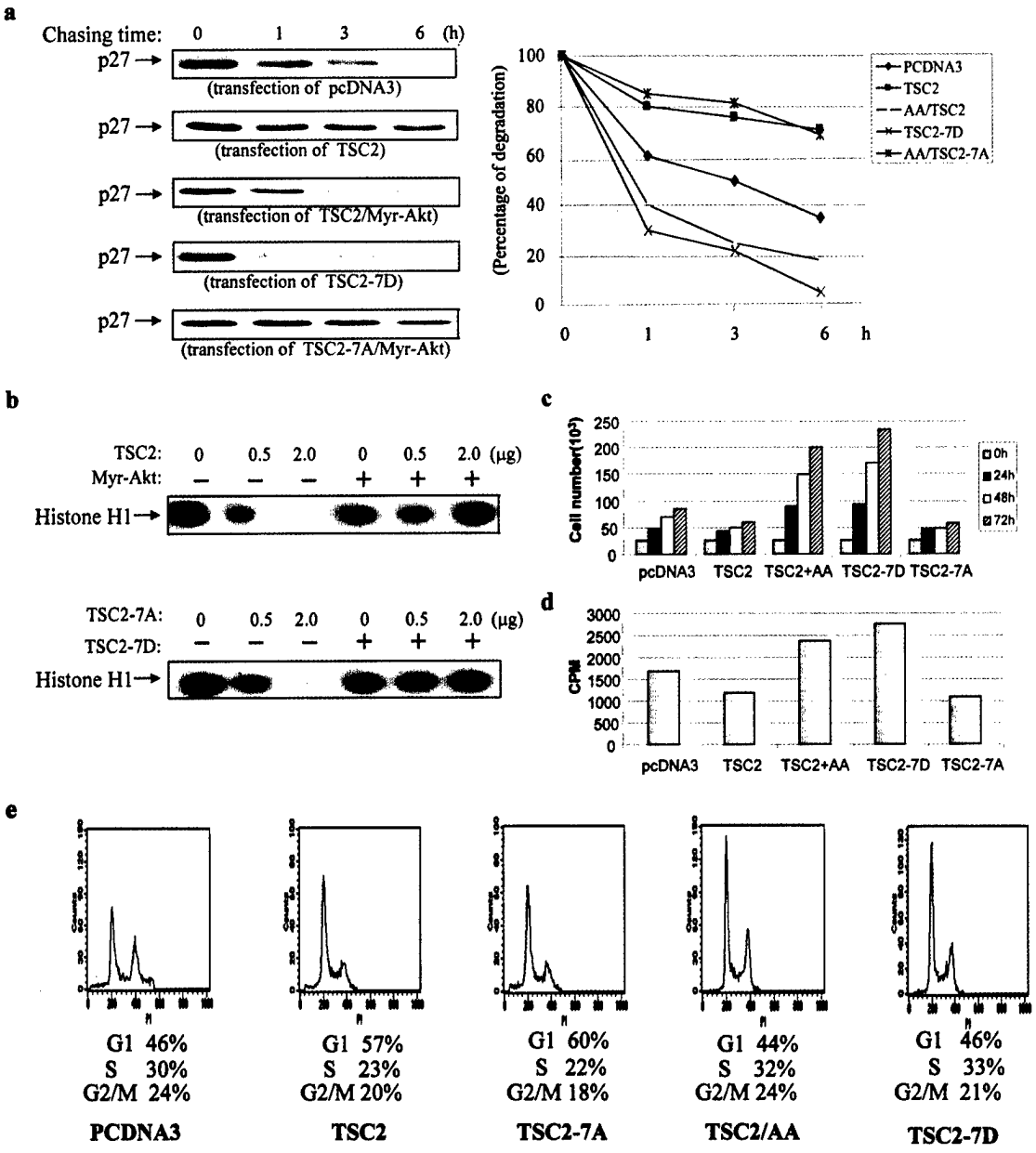

FIG. 4. **Akt phosphorylation of tuberin results in $p27^{kip1}$ degradation and cell proliferation.** *a*, similar to Fig. 4*d*, HeLa cells were transfected with expression constructs indicated at the *bottom* of each panel, labeled with [^{35}S]methionine, chased at indicated times, immunoprecipitated with anti-$p27^{kip1}$ antibody, and separated by SDS-PAGE. Graphical presentations show the normalized density of $p27^{kip1}$ degrading from 100%. *b*, *in vitro* CDK2 kinase assay analyses of cyclin E-CDK2 immunoprecipitates prepared anti-cyclin E antibody from HeLa cells transfected with the indicated plasmids. *c*, cell growth. EEF8 TSC2-deficient cells were transfected with the indicated expression constructs (*bottom of diagram*) in triplicate 6-well plates. Cells were counted using a Coulter cell counter at different times. *d*, thymidine incorporation. EEF8 TSC2-deficient cells were transfected with the indicated plasmid (*bottom of diagram*) and labeled with [^{3}H]thymidine for 12 h. Data were obtained from four independent experiments. *e*, flow cytometry. HeLa cells were co-transfected with CD20 and the expression constructs indicated at the *bottom*. After 48 h, cells were harvested, stained with anti-CD20-FITC antibody and propidium iodide, and analyzed by a fluorescence-activated cell sorter.

tional modification mechanism in which the proteosome pathway is involved.

To examine whether Akt-induced hamartin-tuberin degradation depends upon Akt phosphorylation of tuberin, EEF8 TSC2-deficient cells were transfected with wild type TSC2, phosphomimic TSC2-7D, or nonphosphorylatable TSC2-7A. Western blotting and pulse-chase analyses revealed that TSC2-7D was degraded more rapidly than wild type TSC2, whereas TSC2-7A became more stable. Expression of TSC2-7D promoted hamartin degradation, whereas TSC2-7A stabilized hamartin and inhibited Akt-induced hamartin degradation (Fig. 3*d*), indicating that Akt phosphorylation of tuberin is required for degradation of the hamartin-tuberin complex. Previous studies have shown that tuberin functions as a cytosolic chaperone protein to prevent hamartin self-aggregation and maintain the tuberin-hamartin complex in a soluble form (9, 19). However, we did not observe that Akt phosphorylation of tuberin affected its chaperone function (data not shown).

Akt Phosphorylation of Tuberin Leads to Down-regulation of $p27^{kip1}$ and Cell Proliferation—The results from studying altered expression of either *TSC1* or *TSC2* have demonstrated that both hamartin and tuberin inhibit cell growth and cell size in mammalian (4, 5) and *Drosophila* (10–12), respectively. The mechanism that has been characterized so far is that overexpression of hamartin and tuberin induces the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ through inhibition of

its degradation (20, 21). To examine the effects of Akt phosphorylation of tuberin on $p27^{kip1}$ expression, pulse-chase analyses were performed with TSC2-deficient EEF8 cells that were transfected with TSC2, TSC2/Mry-Akt, TSC2-7D, or TSC2-7A. As shown in Fig. 4*a*, expression of constitutively active Akt abrogated the ability of stabilization of $p27^{kip1}$ by tuberin. $P27^{kip1}$ degraded rapidly in phosphomimic TSC2-7D-transfected cells as compared with the cells expressing wild type TSC2/constitutively active Akt. In contrast, the cells expressing TSC2-7A exhibited similar degradation rate of $p27^{kip1}$ to wild type TSC2-transfected cells. Moreover, expression of TSC2-7A abrogated constitutively active Akt-induced $p27^{kip1}$ degradation (Fig. 4*a*). These data indicate that degradation of $p27^{kip1}$ is regulated by Akt phosphorylation of tuberin.

Because G_1/S CDK 2 is a major target of $p27^{kip1}$ (21), we next examined whether Akt overrides tuberin-inhibited CDK2 activity. Consistent with previous reports (21, 22), expression of wild type *TSC2* inhibited CDK2 activity in a dose-dependent manner. However, constitutively active Akt abrogated *TSC2*-inhibited CDK2 activity (Fig. 4*b*). Phosphomimic TSC2-7D lost the ability to inhibit CDK2 activity, whereas expression of nonphosphorylatable TSC2-7A displayed the same effects as wild type TSC2 (Fig. 4*b*).

Because CDK2 is a major regulator of cell growth and G_1/S transition of the cell cycle, we further examined the effects of Akt phosphorylation of tuberin on cell proliferation measured by cell growth and thymidine incorporation. As shown in Fig. 4, *c* and *d*, expression of TSC2 or TSC2-7A in TSC2-deficient EEF8 cells inhibited cell growth and DNA synthesis as compared with the cells transfected with vector alone. However, cells expressing constitutively active Akt or phosphomimic TSC2-7D significantly enhanced cell growth and thymidine incorporation. Consistent with previous findings, the number of cells at the G_0-G_1 phase of the cell cycle was increased in the cells expressing TSC2. Expression of TSC2-7A displayed a similar effect on cell cycle. In contrast, constitutively active Akt overrode wild type TSC2 action. The cell number of the S phase was increased in constitutively active Akt/TSC2- or phosphomimic TSC2-7D-transfected cells (Fig. 4*e*). These data indicate that Akt phosphorylated tuberin lost its tumor suppressor function at least in part by inducing $p27^{kip1}$ degradation.

DISCUSSION

Recent studies have demonstrated that phosphorylation of hamartin and/or tuberin may play an important role in the formation of the tuberin-hamartin complex. Tuberin is phosphorylated at serine and tyrosine residues, and a disease-related TSC2 tyrosine 1571 mutation (Y1571H) nearly abolishes tuberin tyrosine phosphorylation and disrupts tuberin-hamartin binding, implying that the phosphorylation of tyrosine 1571 of TSC2 is required for tuberin-hamartin complex formation (8, 9). Our study, however, shows that phosphorylation of tuberin by Akt and mitogenic factors (insulin and IGF1) abrogates hamartin-tuberin tumor suppressor activity without interfering with binding but by inducing degradation of both proteins through the proteosome pathway. Therefore, we provide a new paradigm for regulation of the TSC1/TSC2 tumor suppressor pathway.

In addition to the Forkhead transcription factor family (16, 22), tuberin is the second Akt downstream target that has been uncovered by genetic studies so far. In this study, we present molecular evidence that tuberin is a direct physiological substrate of Akt by demonstrating that Akt binds to and phosphorylates tuberin. It has been documented that Akt induces cell cycle progression and cell proliferation through transcription repression and degradation of $p27^{kip1}$ (23, 24). Akt inhibition of $p27^{kip1}$ transcription is achieved by Akt phosphorylation of a

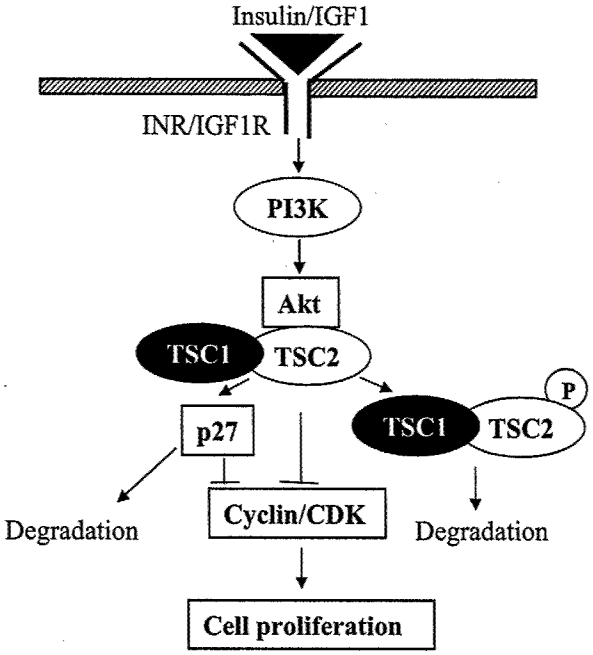

FIG. 5. **Schematic illustration of negative regulation of the TSC tumor suppressor complex by PI3K/Akt.**

Forkhead transcription factor, AFX, leading to the decrease of $p27^{kip1}$ promoter activity (24). However, the mechanism of Akt degradation of $p27^{kip1}$ is unclear. Tuberin was revealed to stabilize $p27^{kip1}$ by maintaining $p27^{kip1}$ in the nucleus (20). We observed in this study that Akt attenuates the tuberin action but does not induce translocation of $p27^{kip1}$ from nuclear to cytoplasm (data not shown). Previous studies have shown that three isoforms of Akt share almost the same upstream regulators and downstream targets. Similarly, we have observed that Akt1, Akt2, and Akt3 all phosphorylate and interact with tuberin, even though Akt2 displays a slightly higher binding affinity to tuberin. The model in Fig. 5 illustrates the mechanism through which the PI3K/Akt pathway mediates insulin and IGF1 signals to down-regulate hamartin-tuberin function by phosphorylation of tuberin. Our results define a possible new mechanism through which Akt induces cell proliferation and transformation by inhibiting TSC1/TSC2 tumor suppressor functions.

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DRUG RESISTANCE Updates

Review

Role of X-linked inhibitor of apoptosis protein in chemoresistance in ovarian cancer: possible involvement of the phosphoinositide-3 kinase/Akt pathway

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Abstract

Although cisplatin derivatives are first-line chemotherapeutic agents for the treatment of epithelial ovarian cancer, chemoresistance remains a major hurdle to successful therapy and the molecular mechanisms involved are poorly understood. Apoptosis is the cellular underpinning of cisplatin-induced cell death, which is associated with expression of specific "death" genes and down-regulation of "survival" counterparts. The X-linked inhibitor of apoptosis proteins (Xiap), an intracellular anti-apoptotic protein, plays a key role in cell survival by modulating death signaling pathways and is a determinant of cisplatin resistance in ovarian cancer cells in vitro. This review focuses on the role of Xiap and its interactions with the phosphoinositide-3 kinase (PI3K)/Akt cell survival pathway in conferring resistance of ovarian cancer cells to chemotherapeutic agents and discusses potential therapeutic strategies in overcoming chemoresistant ovarian cancer.

Keywords: Xiap; PI3K/Akt; Ovarian cancer; Chemoresistance

1. Introduction

Ovarian cancer is the leading cause of cancer death in women in the Western world and ranks fifth among the most common female cancers. The lifetime risk of developing ovarian cancer is 1 in 70 and a lifetime chance of dying of ovarian cancer is 1% (Reis et al., 2000; American Cancer Society, 1997). Epithelial ovarian cancer constitutes approximately 90% of all human ovarian malignancies and originates from the simple epithelium covering the surface of the ovary (Auersbergs et al., 2001; Eltabbakh and Awtrey, 2001). Due to the tendency of most malignant ovarian tumors to insidiously spread within the abdominal cavity by seeding, they are often detected at advanced stages of the disease for which definite cure rates are often low. Indeed, only 25% of patients diagnosed with ovarian cancer have the disease localized in the ovary and the cure rate for advanced stages of cancers (stages III and IV) is below 30%. While over 50% of all ovarian cancer patients will now survive 5 years, cure rates have remained basically the same over the last 20 years (Reis et al., 2000; American Cancer Society, 1997).

Cisplatin and its analogues have been most frequently used for treatment of human cancer, including ovarian cancer. While adjuvant chemotherapy with paclitaxel and cisplatin or carboplatin achieves clinical response in approximately 80% of patients, the tumor recurs in most patients within 3 years following treatment (Eltabbakh and Awtrey, 2001). The overall 5-year survival rate for advanced ovarian cancer patients is still low (20–30%) and is due to chemoresistance in the primary or recurrent tumors, thus resulting in treatment failure. Similarly, although ovarian cancer patients show high response rates to initial chemotherapy after cytoreductive surgery, most patients also develop resistance to chemotherapy during treatment (Katsaros et al., 1999). Overcoming drug resistance is the

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key to successful treatment of ovarian cancer (Kikuchi, 2001).

While the clinical and histological prognostic factors (e.g. surgical stage and tumor grade) are well understood, less is known about the biological process leading to uncontrolled cellular growth. It is now widely accepted that tissue growth is not only dependent on cell proliferation, but also on the rate of apoptosis. Imbalance between these processes leads to uncontrolled tissue growth (Kerr et al., 1994; Reed, 1994; Steward, 1994; Thompson, 1995). The progression of the tumor is, in part, due to the failure of the cells to undergo apoptosis in response to a death signal (Naik et al., 1996). Thus, it is generally accepted that carcinomas may be caused or promoted by factors inhibiting cell death as well as those enhancing cell survival. Whereas the action of cisplatin is thought to be associated with its ability to form inter- and intra-strand DNA cross-links (Li et al., 2000), recent reports suggest that apoptosis may be the cellular underpinning of cisplatin-induced cell death (Li et al., 2000; Sasaki et al., 1999; Arts et al., 2000) and that the DNA-damaging effects of cisplatin are also associated with expression of specific death genes and down-regulation of "survival" counterparts (Li et al., 2000, 2001; Sasaki et al., 1999; Arts et al., 2000; Schneiderman et al., 1999).

The molecular mechanism of chemoresistance in ovarian cancer is multifactorial and poorly understood. It is recognized that 75% of patients resistant to cisplatin treatment also developed resistance to taxol, implying that the resistance to these agents may involve similar mechanisms. Chemoresistance may generally be classified into acquired and de novo forms and has traditionally been thought of in terms of altered drug transport and gene expression (e.g. multi-drug resistance gene), modified drug target, increased rate of DNA repair or decreased rate of drug-induced DNA or macromolecule damage (Reed et al., 1996). A growing body of evidence indicates that defects in the intra- and extra-cellular apoptotic pathways are an important cause of resistance to cytotoxic agents (Arts et al., 2000). Indeed, our recent studies indicate that homeostasis of human ovarian surface epithelial cells is maintained by a delicate balance in the expression and actions of tumor suppressors (e.g. p53, Fas and FasL) and cell survival factors (e.g. inhibitor of apoptosis proteins (IAPs) and intermediates of the PI3K/Akt cell survival pathway). IAPs are suppressors of apoptosis and believed to be determinants of chemosensitivity of ovarian cancer. Moreover, the intermediates of the PI3K/Akt survival pathway are frequently altered in human ovarian cancer (Shayesteh et al., 1999; Cheng et al., 1992; Yuan et al., 2000; Sun et al., 2001a; Philp et al., 2001) and elevated levels of PI3K and AKTs were shown to be associated with poor prognosis and chemoresistance in this malignancy (Sun et al., 2001a; Philp et al., 2001; Page et al., 2000). Chemoresistance in ovarian cancer results in part from over-expression of IAPs, PI3K, and/or AKTs and down-regulation of Fas and FasL, leading to suppressed apoptosis (Li et al., 2000, 2001; Sasaki et al., 1999; Schneiderman et al., 1999).

2. Xiap: a determinant in chemosensitivity in ovarian cancer

The progression of the tumor is, in part, due to suppressed apoptosis (Naik et al., 1996). It has been shown that environmental stress (e.g. DNA damage, drug-induced cell-cycle arrest) elicits rapid cellular responses (e.g. expression of oncogenes and DNA-repair enzymes) for homeostatic maintenance. Thus, it is conceivable that some "cell survival" factors (cell death inhibitors) may be induced and play a promoting role in this process. While expression of *bcl-2* is an important factor in drug-induced apoptosis in ovarian cancer thereby contributing to chemoresistance (Eliopoulos et al., 1995; Herod et al., 1996), evidence also indicates that *bcl-2* over-expression cannot adequately account for the etiology of drug resistance in ovarian cancer (Steward, 1994; Diebold et al., 1996). The IAPs are potential candidates involved in the suppression of apoptosis and in ovarian cancer progression.

The IAPs are intracellular anti-apoptotic proteins, first identified in baculovirus, which play a key role in cell survival by modulating death-signaling pathways. They currently include X-linked IAP (Xiap), human IAP-1 (Hiap-1), human IAP-2 (Hiap-2), neuronal apoptosis inhibitory protein (Naip), survivin and Livin (Fig. 1) (Ambrosini et al., 1997; Liston et al., 1996; Kasof and Gomes, 2001; Lin et al., 2000). These proteins are characterized by the presence of a caspase-recruitment domain (CARD) and an N-terminal baculovirus-inhibitor-of-apoptosis-repeat (BIR) motif, which is necessary for biological activity. With the exception of Naip and survivin, IAPs also contain a C-terminal RING-Zinc finger domain believed to be involved in protein–protein and protein–nucleic acid interactions. Importantly, a recent study reported that the RING finger domain of Xiap and Hiap-1 has ubiquitin protease ligase (E3) activity and is responsible for the autoubiquitination and degradation of IAPs after apoptosis stimulus (Yang et al.,

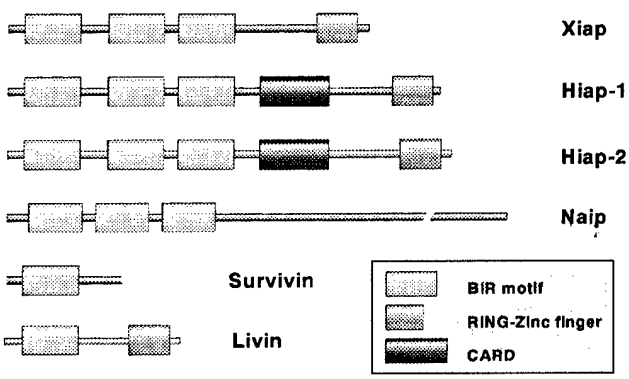
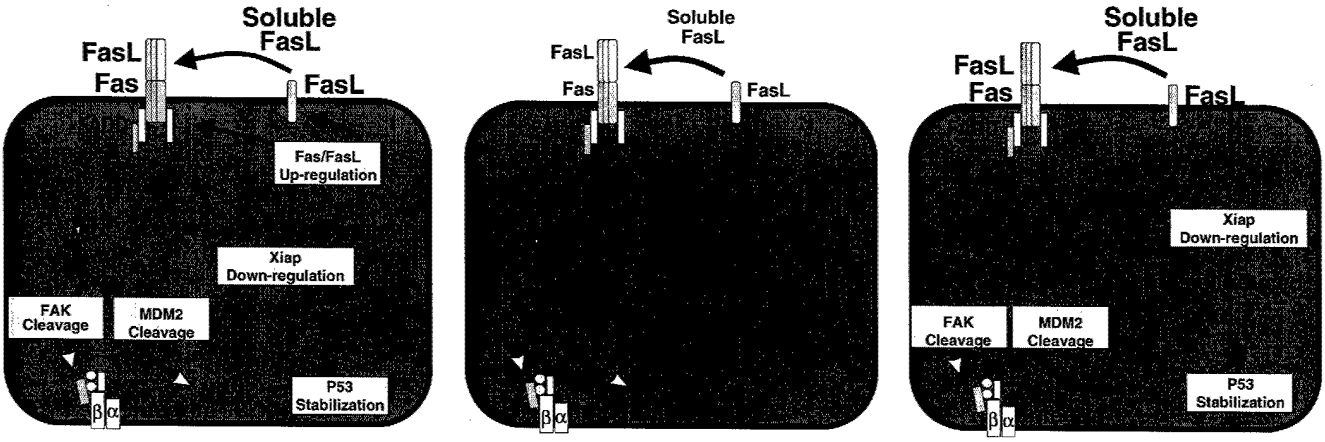


Fig. 1. Diagrammatic representation of the structure of mammalian IAP family members. All IAPs have one or more baculovirus inhibit or of apoptosis repeat (BIR) motifs required for biological activity. Apart from Naip and Survivin, they have RING Zing finger involved in protein–protein interactions and auto-ubiquitination. Hiap-1 and Hiap-2 also have caspase recruitment domain (CARD).

2000). Only a few reports to date have addressed the mechanisms of action of these anti-apoptotic proteins. Hiap-1 and Hiap-2 have been suggested to suppress TNF receptor signaling by binding to TNF-receptor-associated factor. Xiap is a direct inhibitor of caspase-3 and caspase-7 and modulates the Bax/cytochrome c pathway by inhibiting caspase-9 (Takahash et al., 1998; Deveraux et al., 1999, 1998). The anti-apoptosis effects of mammalian IAPs have been well demonstrated, although to different extent depending on the specific apoptosis inducer, cell type and IAP examined (Liston et al., 1996; Uren et al., 1996; Duckett et al., 1996).

The role of Xiap in the regulation of apoptosis in human ovarian cancer has recently been examined. Xiap has been localized in human ovarian carcinomas, with levels being highest in proliferative but not apoptotic epithelial cells (Li et al., 2001). In contrast, expression of Hiap-1, Hiap-2 and Naip is relatively low in these cells. In addition, in vitro studies with cisplatin-sensitive ovarian cancer cell lines (OV2008 and A2780s) and their resistant variants (C13 (with wild type p53) and A2780cp (with mutated p53), respectively) (Liu et al., 1994; Delmastro et al., 1997; Behrens et al., 1987; Andrews et al., 1992) have shown that cisplatin treatment induced a rapid (within 3 h) increase in the Xiap protein content but decreased the Xiap level and induced apoptosis in sensitive cells but not their resistant variants with continuous drug challenge (Figs. 2A and B, Li et al., 2001; Asselin et al., 2001). The differences in the apoptotic response of these cell lines to cisplatin could not be attributed to the relative levels of cisplatin retention (Zinkewich-Peotti and Andrews, 1992). Direct evidence for an important role of Xiap in the regulation of apoptosis in ovarian cancer cells came from experiments in which down-regulation of Xiap by adenoviral Xiap antisense cDNA expression in the cisplatin-sensitive cells in the absence of the chemotherapeutic agent, induced caspase-3 and -9 cleavage and apoptosis (Li et al., 2001; Asselin et al., 2001). More importantly, cisplatin-induced apoptosis in cisplatin-sensitive cells was suppressed by adenoviral Xiap sense expression. These studies strongly suggest that Xiap is a determinant of cisplatin-sensitivity in human ovarian cancer cells and that cisplatin-resistance in human ovarian cancer may in part be due to an inability of this chemotherapeutic agent to decrease Xiap protein content and induce apoptosis (Li et al., 2000, 2001; Asselin et al., 2001). This concept is supported by recent observations on the anti-apoptotic role of Xiap in an oral carcinoma cell line (Matsumiya et al., 2001).

The mechanism by which cisplatin decreases Xiap level in ovarian cancer cells is not fully understood. We have recently shown that cisplatin can decrease Xiap protein content in chemosensitive ovarian cancer cells without a significant change in Xiap mRNA abundance (Asselin et al., 2001), suggesting that *Xiap* gene transcription is not involved in Xiap down-regulation by cisplatin. This notion is consistent with the recent finding that Xiap in thymocytes is degraded in a proteasome-dependent manner in response to the pro-apoptotic action of glucocorticoids or etoposide. Furthermore, Xiap has ubiquitin ligase (E3) activity at the zinc RING finger domain and is able to catalyze its own ubiquitination (Yang et al., 2000). In this regard, over-expression of a zinc RING finger domain deleted-Xiap is more effective than of the wild-type Xiap in preventing cisplatin-induced apoptosis in a cisplatin-sensitive ovarian cancer line (Sasaki et al., 2002) and removal of the RING domain of *Drosophila* IAPs enhances their ability to inhibit cell death caused by expression of the cell death-inducing protein Reaper (Yang et al., 2000). These findings raise the interesting possibility that Xiap auto-ubiquitination and degradation may be an important mechanism which regulates the steady-state Xiap level in ovarian cancer cells and determines the sensitivity of the cells to the pro-apoptotic action of cisplatin.



A. Chemosensitive Cells with Cisplatin

B. Chemoresistant Cells with Cisplatin

C. Chemoresistant Cells with Xiap anti-sense & Cisplatin

Fig. 2. Models showing the influence of cisplatin and Xiap anti-sense on chemosensitive and chemoresistant ovarian cancer cells. Xiap maintains cell survival by suppressing caspase-3 activation. As observed in chemosensitive cells treated with cisplatin, Xiap down-regulation in chemoresistant cells by anti-sense expression results in caspase-3 activation, FAK and MDM2 cleavage, increased p53 levels (via stabilization) and apoptosis.

3. Down-regulation of Xiap induces apoptosis and increases cisplatin-sensitvity

Xiap is often considered a final guardian in preventing and regulating apoptotic cell death. Thus, removal of the anti-apoptotic factor would be expected to result in unleashing the apoptotic process, probably mediated by the release of caspase-3 from its inhibition. Indeed, Xiap down-regulation following adenoviral Xiap antisense expression alone resulted in a concentration- and time-dependent decrease in Xiap content and an increase in apoptosis in the wild-type p53, cisplatin-resistant ovarian cancer cell line (C13) as well as it sensitized the cells to the cytotoxic action of cisplatin. The onset of apoptosis following Xiap down-regulation was associated with detectable cleavage of caspase-3 (activation) and MDM2, an oncoprotein that binds to and facilitates ubiquitin-mediated degradation of p53, as well as a significant p53 accumulation (Fig. 2C, Sasaki et al., 2000). In vitro MDM2 cleavage analysis also indicated that the oncoprotein is a substrate for caspase-3. These findings are consistent with the concept that caspase-3 activation following cisplatin challenge results in MDM2 degradation and p53 stabilization and eventually increase in p53 levels and induction of p53-mediated apoptosis (Fig. 2A, Sasaki et al., 2000).

It is of interest to note that the apoptotic response to Xiap down-regulation was not observed in p53-mutated (A2780-cp) or null (SKOV3) cell lines. While wild-type p53 expression in these genetically deficient cells led to significant apoptosis, co-expression of Xiap antisense and wild type p53 sense in these cells not only resulted in a higher p53 content but also was more effective in eliciting the apoptotic response than wild-type p53 restoration alone (Sasaki et al., 2000). These results are corroborated by the finding that the responsiveness of ovarian cancer to cisplatin is dependent on the normal function of p53 (Laframboise et al., 2000). Taken together, these observations provide strong support for a central role for Xiap in the control of p53 accumulation in ovarian cancer cells, a phenomenon associated with the inhibitory action of Xiap on caspase-3 activity, and provide a framework for novel therapeutic approaches in the treatment of chemoresistant ovarian cancer.

Recent reports have shown that IAPs may play an important role in the control of both receptor-mediated cell survival and death signaling by directly inhibiting downstream caspases (Sasaki et al., 2002; Roy et al., 1997; Deveraux et al., 1997). Several DNA damaging agents, including cisplatin, can up-regulate the expression of Fas (a 45 kDa cysteine-rich transmembrane glycoprotein belonging to the TNF/NGF receptor super-family (Itoh et al., 1991)) and its ligand (FasL) in a variety of tumor cell types including human ovarian cancer cells (Uslu et al., 1996; Muller et al., 1997; Schneiderman et al., 1999). Activation of Fas following Fas ligation leads to the initiation of the apoptotic process, which involves the activation of caspase-8 and caspase-3. While cisplatin is effective in up-regulating Fas expression in cisplatin-sensitive (OV2008 and A2780-s) and resistant (C13) ovarian cancer cell lines and activation of the Fas receptor with a monoclonal activating anti-Fas antibody following the cisplatin challenge induces apoptosis, the apoptotic response in the resistant cells were considerably lower compared to its sensitive counterpart (Schneiderman et al., 1999). Interestingly, cisplatin was effective in inducing the cleavage (activation) of caspase-8 and -3 in the sensitive but not resistant cells. These results are in accordance with a central modulatory role of Xiap as a caspase inhibitor and in Fas-mediated death signaling in cisplatin-resistance in ovarian cancer cells.

Recent studies have shown that cell adhesion is an important cell survival determinant and that the loss of cell–cell or cell–matrix contact, known as *anoikis* (Greek for homelessness), induces apoptosis (Frisch and Francis, 1994). While cancer cells are capable of invading surrounding tissue and implanting in distant site via blood stream or peritoneal dissemination, derangement of cell adhesion molecules has been reported in various cancer cells, including those of the ovary (Buczek-Thomas et al., 1998). Cisplatin-induced apoptosis is believed to be cell density-dependent (Takemura et al., 1991), although the precise mechanism of this phenomenon and the effect of cisplatin on cell adhesion are not fully understood. Focal adhesion kinase (FAK), a 125-kDa non-receptor protein tyrosine kinase involved in integrin-mediated cell adhesion, is a downstream effector of the integrin signaling pathway. Upon association with integrin, FAK is autophosphorylated and activated and apoptosis is suppressed. The anti-apoptotic action of FAK is mediated via its binding to other signal transduction molecules required for activation of the Ras-mitogen activated protein kinase (MAPK) cascade and of the PI3K/Akt pathway or cytoskeletal proteins in the formation of focal adhesion. It has been demonstrated that FAK suppresses anchorage-dependent apoptosis (Frisch et al., 1996) and that inhibition of FAK induces apoptosis (Hungerford et al., 1996) and reduces cell motility (Ilic et al., 1996). FAK is proteolytically cleaved during apoptosis induction and caspases have been suggested to be involved (Crouch et al., 1996).

Similarly, the possible role of FAK processing and its regulation in a cisplatin-sensitive human ovarian epithelial cancer cell line (OV2008) have been examined (Sasaki et al., 2002). Treatment of cancer cells with cisplatin results in caspase-3-mediated FAK cleavage, cell detachment from the growth surface and apoptosis. Addition of active caspase-3 to the whole cell lysate elicited a similar pattern of FAK cleavage. FAK fragments were detected exclusively in cells detached from the growth surfaces. Furthermore, inhibition of caspase-3 attenuated cisplatin-induced FAK cleavage and apoptosis. FAK processing is modulated by Xiap and may play an important role in cell detachment and termination of the integrin-mediated anti-apoptotic signal. These findings support the hypothesis that Xiap-mediated resistance of ovarian cancer cells to cisplatin may in part be due to Xiap

suppression of FAK processing and ultimately the maintenance of cell–matrix interaction (Fig. 2A and B, Sasaki et al., 2002).

4. Endogenous regulators of Xiap function

The caspase-inhibiting activity of Xiap is negatively regulated by at least three intracellular Xiap-interacting proteins: Xiap-associated factor 1 (XAF1), DIABLO/Smac and HtrA2/Omi (Liston et al., 2001; Srinivasula et al., 2000; Du et al., 2000; Verhagen et al., 2000; Suzuki et al., 2001). XAF1 is a nuclear protein that directly inhibits the anti-caspase activity of Xiap. XAF1 resides in the nucleus and can effect the re-localization of either endogenous or over-expressed XIAP protein from the cytoplasm to the nucleus. It functions as a negative regulator of the IAPs (Liston et al., 2001). Xiap suppresses caspase activation and cell death in vitro, and XAF1 antagonizes these activities. XAF1 is either not expressed, or present only in low levels in most cancer cell lines, suggesting that alterations in the balance of IAP and caspase activities is a common occurrence in the development of the transformed state. It has also been proposed that limited activation of caspases may be required for some normal cellular processes, such as T-cell activation, and may be initiated via tightly controlled expression of XAF1 (Liston et al., 2001). XAF1 does not require an activation signal, and appears to constitutively interact with Xiap and inhibit its function in healthy cells.

DIABLO/Smac is a mitochondrial protein that is released into the cytosol in response to apoptotic triggers. The protein is able to promote cytochrome c-mediated apoptosis by binding to and inhibiting Xiap (Du et al., 2000; Verhagen et al., 2000). Unlike XAF1, the functional domains of DIABLO/Smac have been established (Srinivasula et al., 2000; Du et al., 2000). Amino terminal sequences in DIABLO/Smac are required for Xiap inhibition. Indeed, mutation of the very first amino acid abolishes DIABLO/Smac function (Du et al., 2000). While BIR3 and BIR2 of Xiap are associated with caspase-9 and caspase-3 inhibition, respectively, four NH_3 terminal amino acids (Ala-Val-Pro-Ile) in DIABLO/Smac interact with the BIR3 domain of Xiap. Furthermore, wild-type DIABLO/Smac can act as a dimer to bind the BIR2 domain of Xiap. Over-expression of either DIABLO/Smac or XAF1 does not appear to induce apoptosis, but does sensitize cells to additional cell death triggers (Liston et al., 2001).

More recently, a serine protease, called HtrA2/Omi, has been reported to be released from the mitochondria and to inhibit Xiap function in a manner similar to that of DIABLO/Smac (Suzuki et al., 2001). Moreover, when overexpressed outside mitochondria, HtrA2/Omi induces atypical cell death, which is neither accompanied by a significant increase in caspase activity nor inhibited by caspase inhibitors, including Xiap (Suzuki et al., 2001). These interesting findings support the contention that in addition to its role as a caspase activator, HtrA2/Omi is also a caspase-independent cell death inducer, the action of which involves serine protease activation.

5. FLIP expression and resistance to TNFα- and trail-induced apoptosis

Tumor necrosis factor-α (TNFα) is both a death and survival factor in various cell lineages (Baker and Reddy, 1996), but the molecular and cellular basis for this phenomenon is not completely understood. The action of TNFα is mediated by its receptors, TNFR1 and TNFR2, and binding of TNFα to its receptors activates caspase-8 and caspase-3 (Boldin et al., 1996; Chinnaiyan et al., 1996; Medema et al., 1997; Yang et al., 1998) as well as induces IκB phosphorylation and degradation, and activates NFκB (Kruppa et al., 1992; Laegreid et al., 1994; Rothe et al., 1994; Berberich et al., 1994; Sarma et al., 1995).

It has been demonstrated that the inability of TNFα to induce apoptosis is due to the induction of survival factors, including IAPs (Erl et al., 1999; Stehlik et al., 1998; Xiao et al., 2001) and members of the Bcl-2 family (Grimm et al., 1996; Zong et al., 1999). Although Xiap is important in determining the apoptotic responsiveness of ovarian granulosa cells to TNFα (Xiao et al., 2001), it appears to play a minimal role, if any, in conferring resistance of the human ovarian cancer cell OV2008 to the cytotoxic action of the cytokine, as TNFα decreases Xiap content in the ovarian cancer cells. However, it is of interest to note that, in the presence of protein synthesis inhibitor CHX, TNFα induced Xiap cleavage in OV2008 cells, a process sensitive to the presence of the caspase inhibitors ZVAD and DEVD. These findings, together with the observations that cleavage of Xiap produces an N-terminal BIR-2 fragment with reduced ability to inhibit caspase-3 and suppress apoptosis (Deveraux et al., 1999), support the contention that the caspase-3-mediated processing of Xiap may in part be involved in the execution of apoptosis in ovarian cancer cells in response to TNFα.

Flice-like inhibitory protein (FLIP) is an intracellular anti-apoptotic protein, which modulates the cell surface receptor-mediated cell death process by inhibiting the activation of caspase-8 (Kreuz et al., 2001; Micheau et al., 2001). FLIP is a FADD-binding suppressor of apoptosis and is present as long ($FLIP_L$) and short ($FLIP_S$) isoforms. Both isoforms contain two death effector domains (DED), a structure resembling the N-terminal half of caspase-8 (Thome et al., 1997; Hu et al., 1997; Goltsev et al., 1997). Through DED–DED interaction, FLIP functions as dominant negative for FLICE, and blocks Fas-mediated apoptosis by preventing the activation of caspase-8 (Hu et al., 1997; Irmler et al., 1997; Srinivasula et al., 1999). Recent studies have demonstrated a role of FLIP and regulation of its expression by TNFα in a human ovarian cancer cell line (OV2008) in vitro. While TNFα alone was incapable of

inducing cell death, it remarkably increased the apoptotic cell number, caspase-8 and -3 cleavage in the presence of the protein synthesis inhibitor cycloheximide. TNFα also induced NFκB-mediated expression of $FLIP_S$, but not of $FLIP_L$. While down-regulation of $FLIP_S$ expression by $FLIP_S$ antisense cDNA facilitated TNF-induced cell death, over-expression of $FLIP_S$ sense attenuated TNF-induced apoptosis in the presence of cycloheximide. This study demonstrated that TNFα up-regulates $FLIP_S$ expression and this effect is mediated by the activation of NFκB. The induction of $FLIP_S$ expression by TNFα might contribute to the resistance of OV2008 cells to the pro-apoptotic action of the cytokine (Xiao, C.W., Li, Y., Reddy, S.A.G. and Tsang, B.K., unpublished observations).

It is of interest to note that the role of FLIP in conferring resistance to cell surface receptor-mediated apoptosis is not confined to the action of TNFα. The TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family and an apoptosis inducer in tumor but not normal cells via death receptors DR4 and DR5 (Suliman et al., 2001). The differences in sensitivity of various transformed and cancer cells to the pro-apoptotic action of TRAIL appear to be associated with the differences in $FLIP_L$ expression (Leverkus et al., 2000; Griffith et al., 1998). In addition, antigen receptor signaling is known to up-regulate $FLIP_L$ in primary B cells and to suppress the Fas- and TRAIL-receptor mediated apoptosis (Wang et al., 2000) and Fas-mediated apoptosis associated with the pathophysiology of rheumatoid arthritis is regulated at the level of caspase-8 through increased FLIP expression (Kobayashi et al., 2000). Moreover, c-$FLIP^{-/-}$ embryonic fibroblasts are highly sensitive to FasL- or TNF-induced apoptosis and show rapid induction of caspase activities, suggesting that c-FLIP mediates cytoprotection against death factor-induced apoptosis (Yeh et al., 2000). Whether FLIP has a role in conferring resistance of ovarian cancer cells to the cytotoxic action of TRAIL remains to be determined.

6. PI3K/Akt pathway in cancer cells

Phosphoinositide 3-kinase (PI3K) is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit. In mammalian cells, three isoforms of p85 and p110 have been cloned, namely p85α, p85β, p85γ, p110α, p110β, and p100γ, and found to differ in their activation mechanisms by extracellular agonists, substrate specificity, and subcellular and tissue distribution. PI3K phosphorylates inositol lipids at the 3′ position of the inositol ring to generate the 3-phosphoinositides PtdIns-3-P, PtdIns-3,4-P2, and PtdIns-3,4,5-P3. Type III PI3Ks are responsible for the synthesis of PtdIns-3-P, which is constitutively present in all cells, and its levels do not dramatically change following stimulation. In contrast, PtdIns-3,4-P2 and PtdIns-3,4,5-P3, generated by types I and II enzymes, are normally undetectable in most cells. Their levels rapidly accumulate upon stimulation that activates downstream molecules including pleckstrin homology (PH) domain-containing proteins PDK1 and Akt, as well as PKC, JNK1, and p38 (Leevers et al., 1999) to mediate a variety of cellular responses including cell growth and transformation, differentiation, motility, insulin action, and cell survival (Fig. 3).

Numerous studies have shown that Akt is a major target of PI3K. Akt, also known as protein kinase B (PKB), is a subfamily of serine/threonine protein kinases (Cheng et al., 1992; Bellacosa et al., 1991; Jones et al., 1991a,b; Nakatani et al., 1999). Three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, have been identified in this family. While AKT1 is the true human homologue of v-akt/mouse Akt (98% identity at the amino acid level), AKT2 is a v-akt closely related kinase (Cheng et al., 1992). The three isoforms of Akt/PKB are highly homologous to v-akt. The overall homology of between these three isoforms is >85%. They share a very similar structure, which contains an N-terminal PH domain, a central kinase domain, and a serine/threonine-rich C-terminal region. The PH

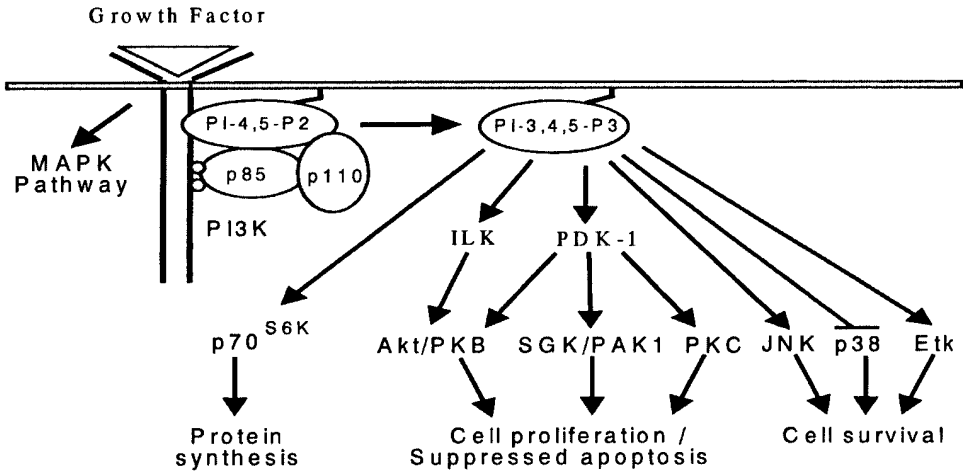


Fig. 3. The PI3K pathway and major downstream targets of PI3K.

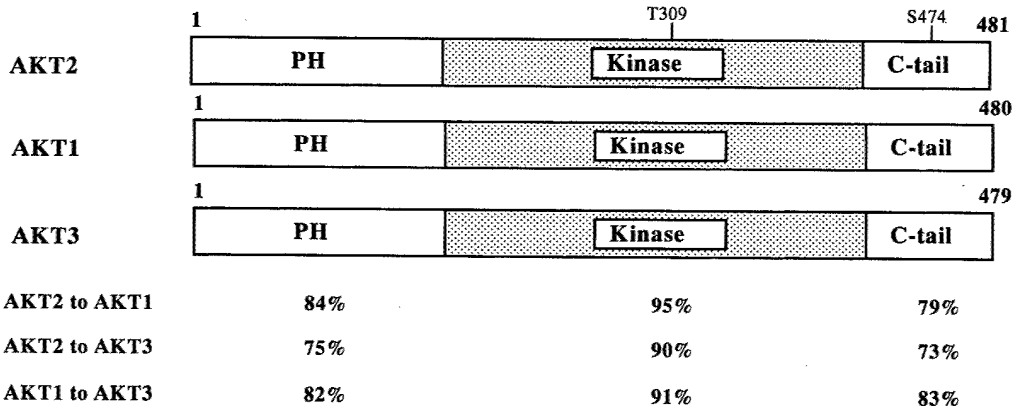


Fig. 4. Diagrammatic representation of the structure of Akt family members. The percentages indicate the degree of homology between AKT1, AKT2 and AKT3 proteins in three different domains: PH, kinase, and regulatory (C-tail) regions.

domain and C-terminal region between these three isoforms are more diverse (homology 73–84%) as compared to the kinase domain (homology 90–95%), suggesting that PH and C-terminal regions may represent functional difference between A/Akt, AKT2 and AKT3 (Fig. 4).

It has been shown that Akt is activated by a variety of stimuli in a PI3K dependent manner and is essential for cell survival (Franke et al., 1995; Burgering et al., 1995; Meier et al., 1997; Liu et al., 1998). Activation of Akt by growth factors depends on the integrity of the PH domain, which binds to PI3K products PtdIns-3,4-P2 and PtdIns-3,4,5-P3, and on the phosphorylation of Thr^{308} (Thr^{309} in AKT2 and Thr^{305} in AKT3) in the activation loop and Ser^{473} (Ser^{474} in AKT2 and Ser^{472} in AKT3) in the C-terminal activation

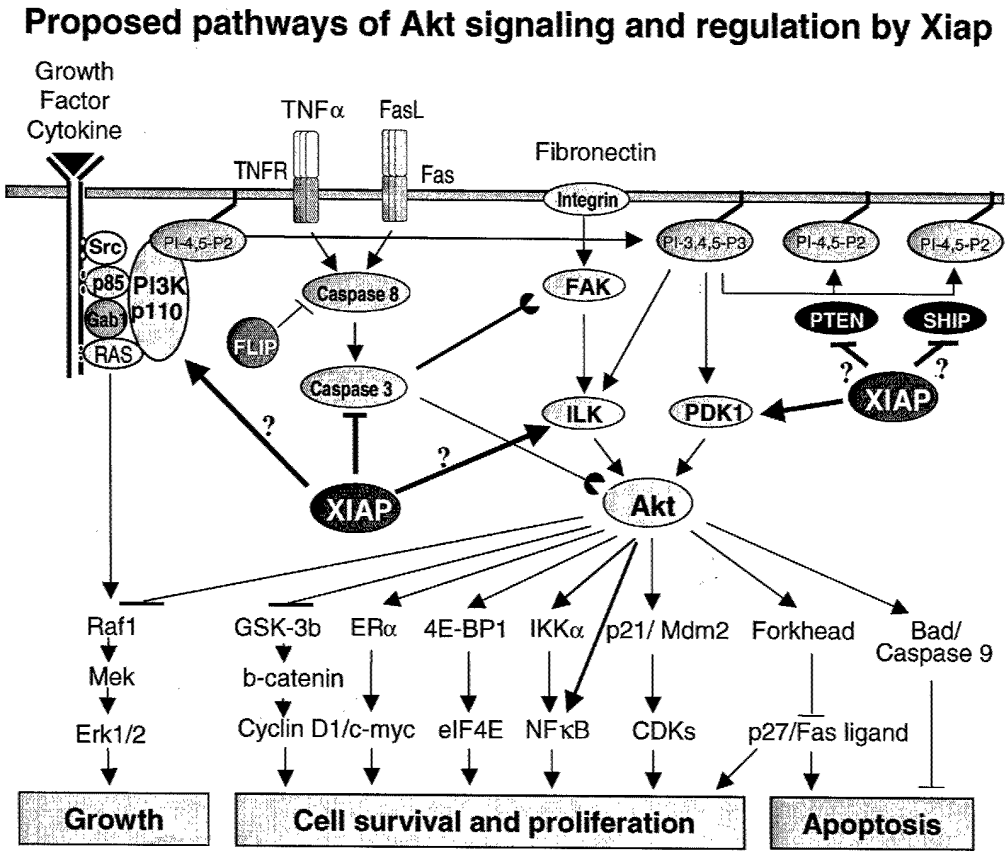


Fig. 5. Proposed pathways of cell signaling and regulation by Xiap. Activation of Akt is mediated by PI3K. In addition, activated Ras, Src and Gabi as well as Xiap have been shown to induce PI3K/Akt pathway. Ligation of Fas and TNF receptor (TNFR) leads to the activation of caspase-8 and caspase-3, and caspase-3-mediated cleavage of FAK and Akt.

domain by PDK1 (Fig. 5, Chan et al., 1999). The activity of Akt is negatively regulated by *PTEN*, a tumor suppressor gene that is mutated in a number of human malignancies including human endometroid ovarian carcinoma. *PTEN* encodes a dual-specificity protein and lipid phosphatase that reduces intracellular levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in cells by converting them to PtdIns-4-P1 and PtdIns-4,5-P2 respectively, thereby inhibiting the PI3K/Akt signaling pathway (Li et al., 1998; Stambolic et al., 1998).

Although AKT1, AKT2, and AKT3 display high sequence homology and share similar upstream signal regulators and downstream targets, there are clear differences between the three isoforms in terms of biological and physiological function: (a) *AKT1* expression is relatively uniform in various normal organs whereas high levels of *AKT2* and *AKT3* mRNA are detected in skeletal muscle, heart, placenta and brain (Cheng et al., 1992; Nakatani et al., 1999; Bellacosa et al., 1993; Altomare et al., 1995); (b) overexpression of wild type AKT2, but not AKT1, transforms NIH3T3 cells (Cheng et al., 1997; Ahmed et al., 1993); (c) AKT2 and AKT3, but not AKT1, are amplified and/or upregulated in certain types of human cancer, such as carcinoma of ovary, breast, and pancreas (Cheng et al., 1992, 1996; DeFeo et al., 1995; Miwa et al., 1996; Ruggeri et al., 1998; Nakatani et al., 1999); (d) AKT1 and AKT3, but not AKT2, are activated by heat shock and H_2O_2 (Shaw et al., 1998); and (e) Akt2- and Akt1-deficient mice displayed different phenotypes. A recent study demonstrated that knockout mice deficient in Akt2 are impaired in the ability of insulin to lower blood glucose because of defects in the action of the hormone on skeletal muscle and liver. $Akt2^{-/-}$ mice are born without apparent defects, but develop peripheral insulin resistance and non-suppressible hepatic glucose production, resulting in hyperglycemia accompanied by inadequate compensatory hyperinsulinemia (Cho et al., 2001a), similar in some important features to type 2 diabetes in human. These phenotypic characteristics are not compensated by the presence of Akt1 and Akt3, reflecting differences of substrate specificity in insulin-responsive tissues. In contrast, *Akt1*-deficient mice did not display a diabetic phenotype (Chen et al., 2001; Cho et al., 2001b). The mice are viable but display impairment in organismal growth, i.e. are smaller when compared to wild type littermates. Such relatively subtle phenotypic change in $Akt1^{-/-}$ mice suggests that Akt2 and Akt3 may substitute to some extent for Akt1 (Chen et al., 2001). Nevertheless, the data indicate that there are non-redundant functions between the three isoforms of Akt in certain tissues and/or cell types.

7. PI3K and Akt in ovarian cancer chemoresistance

Alterations of PI3K in human maligriancies have been demonstrated including carcinomas of ovary, colon, breast and lung (Shayesteh et al., 1999; Yuan et al., 2000; Phillips et al., 1998; Moore et al., 1998; Shaw et al., 1997; Sun et al., 2001b). Among downstream targets of PI3K, only the Akt family has been implicated in malignant transformation (Cheng et al., 1997; Mende et al., 2001). We previously demonstrated alterations of AKT2 at DNA and mRNA levels in 15–20% of human ovarian cancer (Cheng et al., 1992; DeFeo et al., 1995) and recently documented frequent activation/overexpression of AKT2 (36%/46%) in 91 primary ovarian carcinomas by an in vitro kinase assay (Yuan et al., 2000). We have also shown that activation of Akt in human ovarian carcinoma resulted from overexpression and/or activation of PI3K and that inhibition of PI3K induced programmed cell death in ovarian cancer cell lines overexpressing AKT2 (Yuan et al., 2000; Sun et al., 2001a). The majority of cases with PI3K/Akt alterations are high grade and late stage tumors, implying that PI3K/Akt is associated with ovarian cancer progression rather than initiation. These studies suggest that PI3K/Akt pathway may play a pivotal role in ovarian carcinogenesis.

Recent studies indicated that expression of Xiap and HER-2/neu rendered tumor cells resistant to TNFα or chemotherapeutic agents by activation of PI3K/Akt pathway (Asselin et al., 2001; Zhou et al., 2000a). A report further demonstrated that ovarian cancer cells either overexpressing constitutively active Akt/AKT1 or containing *AKT2* gene amplification were far more resistant to paclitaxel than cancer cells expressing low AKT levels (Page et al., 2000). We have recently observed that cisplatin-sensitive ovarian cancer cells (A2780s and OV2008) transfected with constitutively active AKT2 became resistant to cisplatin, whereas overexpression of dominant negative AKT2 rendered cisplatin-resistant ovarian cancer cells (A2780cp and C13) susceptible to cisplatin-induced apoptosis (unpublished data). In addition, we previously reported inhibition of tumorigenicity of pancreatic cancer cell lines by antisense AKT2 (Cheng et al., 1996) and recently demonstrated that PI3K/AKT2 is a critical target for farnesyltransferase inhibitor (FTI)-induced apoptosis and constitutively active PI3K and AKT2 overcame FTI-277-induced programmed cell death (Jiang et al., 2000). Taken together, these data indicate that the PI3K/Akt pathway is a critical target for ovarian cancer intervention and that activation of this pathway is associated with chemoresistance in human ovarian carcinoma. Mechanisms for PI3K/Akt mediated chemoresistance include suppression of apoptosis, cell cycle progression, and angiogenesis.

7.1. Suppression of apoptosis

In numerous cell types, it has been shown that Akt induces survival and suppresses apoptotic death induced by a variety of stimuli. A major identified target of Akt is Bad that is phosphorylated by Akt at Ser-136 (Jiang et al., 2000; del Peso et al., 1997; Datta et al., 1997). It has also been shown that Akt activates PAK1 that in turn phosphorylates Bad at Ser-112 resulting in its release from $Bcl\text{-}_{xL}$ complex.

Furthermore, Akt phosphorylates Forkhead transcription factors and IκB kinase α (IKKα) leading to inhibition of Fas ligand transcription and activation of the NF-κB cascade (Datta et al., 1999). It has also been shown that Akt could target at the postmitochondrial level, as expression of constitutively active Akt failed to inhibit cytochrome c release induced by DNA damaging agents (Zhou et al., 2000b). The mechanism could include either enhancement of Xiap function or inhibition of Xaf-1, DIABLO/Smac or HtrA2 activity. Indeed, our recent data show that activation of Akt induces Xiap expression (see below).

7.2. Cell cycle progression

Akt phosphorylates glycogen synthase kinase (GSK)-3β and ERα resulting in upregulation of c-myc and cyclin D1 (Sun et al., 2001b; Datta et al., 1999). The cyclin-dependent kinase (CDK) inhibitor p27 is also down-regulated by Akt at both the transcription and posttranslation levels (Medema et al., 2000). Moreover, Akt phosphorylates and abrogates p21 function (Rossig et al., 2001). Recent studies also documented that Akt targets MDM2 to the nucleus, resulting in decreases of protein level and transcriptional activity of p53 (Mayo and Donner, 2001).

7.3. Angiogenesis

Accumulated evidence shows that Akt plays a central role in angiogenesis by mediating vascular endothelial growth factor (VEGF) signals and inducing transcription and translation of VEGF. It is well known that VEGF has various functions on endothelial cells, the most prominent of which is the induction of proliferation and differentiation by selectively binding to the Flk-1/KDR receptor and subsequent activation of PI3K/Akt pathway (Gerber et al., 1998). Moreover, constitutively active Akt can induce VEGF mRNA expression by stabilization (Zhong et al., 2000) and enhancing translation (Laughner et al., 2001) of HIF1α, which translocates to the nucleus, dimerizes with UIF1β and activates VEGF transcription.

8. Interactions between Xiap and PI3K pathway as possible mechanisms for chemoresistance

Although PI3K, Akt and Xiap are important cell survival factors in ovarian cancer cells (Li et al., 2001; Sasaki et al., 2000; Chan et al., 1999; Brunet et al., 2001), if and how they interact to confer resistance to chemotherapy is not known. Our laboratories have recently investigated the role of Xiap in the regulation of PI-3Kinase/Akt cell survival pathway in chemosensitive (A2780-s, OV2008 and OVCAR-3) and resistant (A280-cp) ovarian cancer cell lines and the nature of this interaction in cell death/survival signaling (Asselin et al., 2001). During a 24-h culture period, cisplatin decreased Xiap protein levels, activated caspase-3 and -9, and induced Akt cleavage and apoptosis in chemosensitive but not in resistant ovarian cancer cells (Li et al., 2001; Asselin et al., 2001). The Akt cleavage appeared to be mediated through caspase-3 activation, as human recombinant active caspase-3 was able to cleave Akt in vitro. The cisplatin-induced activation of caspase-9 and caspase-3 was blocked by Xiap over-expression (Asselin et al., 2001). Xiap down-regulation by antisense expression also induced Akt cleavage and apoptosis. Pretreatment ovarian cancer cells and their whole cell lysate with tetrapeptide inhibitors of caspase (e.g. DEVD) in vitro significantly decreased Akt cleavage induced by cisplatin and exogenous active caspase-3, respectively (Asselin et al., 2001). These results clearly demonstrate that Xiap plays an important role in the regulation of cisplatin sensitivity of ovarian cancer cells in part by modulating caspase-3-mediated cleavage of Akt, and thus, the integrity and function of the PI 3-kinase/Akt cell survival pathway.

We have also demonstrated that Xiap prevents apoptosis through up-regulation of PI3K/Akt cell survival signaling pathway and that the site of action of Xiap is in part upstream of Akt. Specifically, over-expression of Xiap by adenoviral sense Xiap cDNA infection increased phospho-Akt content (indicative of Akt activation), which was associated with a decrease in cisplatin-induced apoptosis (Asselin et al., 2001). However, in the presence of the PI3K inhibitor LY294002, Xiap over-expression failed to block cisplatin-induced apoptosis and to increase phospho-Akt content (Asselin et al., 2001), suggesting that the site(s) of action of Xiap on the PI3K/Akt pathway is at and/or proximal to PI3K. Whether Xiap up-regulates this pathway by turning on kinases (e.g. PI3K, integrin-linked kinase (ILK) and phosphoinositide-dependent kinase 1 (PDKI)) and/or down-regulating phosphatases (e.g. SH2-containing inositol polyphosphate-5-phosphatase (SHIP) and phosphatase and tensin homology (PTEN)) is not known (Fig. 5). Further, Akt has recently been shown to participate in the regulation of both p53-dependent and p53-independent p21 expression in ovarian cancer cells (Mitsuuchi et al., 2000) and that the induction of apoptosis by antisense Xiap expression is dependent on the p53 status of the cells. However, it is not known whether the regulation of the PI3K/Akt pathway by Xiap and its role to cisplatin-resistance is p53 dependent. Nevertheless, these findings offer a new mechanism by which Xiap may regulate apoptosis and confer cisplatin-resistance in ovarian cancer cells.

In addition, we have recently observed that Akt induces Xiap expression at the protein level in HeLa and ovarian cancer cells transfected with constitutive active Akt in a tetracycline-inducible system. Although the mechanism(s) of this regulation is not known, it may possibly include (a) increased Xiap promoter activity via activation of the NFκB pathway, (b) phosphorylation of $p70^{S6K}$ and 4E-BP1, leading to the induction of Xiap translation and (c) inhibition of Xiap degradation. Nevertheless, Akt-induced Xiap expression supports the notion that Akt exerts its anti-apoptotic

effect at the post-mitochondrial level (Zhou et al., 2000b), and could be a major determinant for activation of PI3K/Akt pathway-associated chemoresistance in human ovarian cancer. The notion that Akt may play a role in the regulation of Xiap expression is supported by our recent observation that the follicle stimulating hormone-induced, NFκB-mediated Xiap expression in rat ovarian granulosa cells is sensitive to the PI3K inhibitor LY294002 in vitro (Wang et al., 2001).

9. Xiap and PI3K/Akt pathway as potential therapeutic targets

As elaborated earlier, the ability of a therapeutic agent to induce apoptosis is a critical factor in the success of ovarian cancer chemotherapy. Although cisplatin and taxol derivatives are first-line chemotherapeutic agents for ovarian cancer, chemoresistance remains a major therapeutic hurdle. The prospect of gene manipulation as an alternative therapeutic approach for ovarian cancer, particularly for the chemoresistant disease, has generated considerable excitement. Adenovirus has been the most promising vehicle for gene replacement, but the use of non-DNA-based viruses is also being explored. Recent novel advances in this therapeutic approach include refinement of vector targeting and the use of site-specific promoters and conditionally replicative adenoviral vectors (Collinet et al., 2000). Of the 380 gene therapy clinical trials (3173 patients) currently in progress or to be initiated, 16 trials are related to ovarian cancer and involve mutation compensation by replacement of an altered tumor suppressor gene, molecular therapy by suicide gene or multi-drug resistance gene transfer, and oncogene inhibition (Collinet et al., 2000). Although several clinical trials have documented the relative safety of gene therapy in ovarian cancer patients, few significant clinical responses have been effected. Targeting Xiap or its function has not been explored.

We have recently shown that down-regulation of Xiap with an adenovirus vector containing a Xiap antisense cDNA sensitizes cisplatin-resistant ovarian cancer cell lines to the pro-apoptotic action of cisplatin (Sasaki et al., 2000). As over 75% of ovarian cancers carry a mutated and non-functional *p53* gene and the induction of apoptosis by Xiap antisense expression is p53-dependent (involves caspase-dependent p53 accumulation), down-regulation of Xiap alone would have limited value as a therapeutic strategy. However, the observation that Xiap down-regulation enhances the apoptotic response induced by adenoviral WT-p53 sense expression raises the interesting possibility that down-regulation of Xiap expression or function could serve as an important adjuvant for *p53* gene therapy (Sasaki et al., 2000). These results provide the necessary proof of principle for Xiap as an important etiological factor in cisplatin resistance in ovarian cancer and demonstrate Xiap may be a target for gene or molecular therapy. It should be noted, however, that antisense expression as an effective treatment modality in vivo is often problematic and unreliable. However, the expression of a dominant negative, which inhibits Xiap function may prove to be a more promising strategy.

Xiap is negatively regulated by at least three endogenous proteins: XAF1, DIABLO/Smac and HtrA2/Omi (Liston et al., 2001; Srinivasula et al., 2000; Suzuki et al., 2001). In vitro studies have shown that the prevention by Xiap of apoptosis in fibroblasts following serum withdrawal was dose-dependently antagonized by the *xaf1* gene, introduced using an adenovirus vector (Liston et al., 2001). It is possible, therefore, that over-expression of the *xaf1* gene may be a more effective mechanism of overcoming Xiap-induced chemoresistance. Introduction of the wild-type full-length XAF1 protein by adenoviral *xaf1* gene delivery may be an option. However, elucidation of the functional components of the protein may allow the design of small peptides containing the XAF1 domains crucial for Xiap sequestration. These small peptides would theoretically possess the same ability to sequester Xiap as the wild-type XAF1 protein, but could be readily taken up by the cells. The recent identification of the functional motif of DIABLO/Smac raises the possibility that small peptides which inhibit either or both of the BIR2 and BIR3 domains of Xiap, may potentially be promising candidates for future anti-cancer agents. In this context, it is of interest to note that a short 7-residue peptide derived from the DIABLO/Smac NH_3-terminal, which can promote the activation of procaspase-3, has already been reported (Wu et al., 2000).

Furthermore, current evidence suggests that overexpression of XAF1 or DIABLO/Smac does not, by itself, induce apoptosis. These factors, however, can sensitize cells to the actions of apoptotic stimuli. In this regard, using these peptides as models for novel anti-cancer agents may prove useful in that they are not expected to cause unwanted cell death in the absence of a chemotherapeutic agent. The therapeutic potential could possibly be realized with heightened sensitivity to chemotherapy following targeting of the exogenous peptide with cell-specific promoters. The search for ovarian cell-specific promoters for ovarian cancer is on-going and the recent demonstration that OSP-1 promoters can successfully drive the expression of a *lacZ* gene in an ovarian-specific manner is particularly noteworthy (Garson et al., 2001; Selvakumaran et al., 2001).

Similar to DIABLO/Smac and XAF1, Omi/HtrA2 has been identified as a direct Xiap binding protein (Suzuki et al., 2001). Whether overexpression of all or part of the HtrA2 protein, either by genetic manipulation or by introduction of a small peptide containing the functional Xiap inhibition domain, can serve as a useful adjuvant to traditional chemotherapy in chemoresistant ovarian cancer remains to be determined. In addition, the importance of the serine protease-containing domain of HtrA2 in caspase-independent cell death induction and as a potential candidate for a therapeutic agent for ovarian cancer remains to be explored.

While it is known that Xiap may act through caspase inhibition and/or through the PI3K/Akt pathway, the relative contribution of each of these pathways to chemoresistance is not known. As discussed in the previous section, PI3K/Akt pathway is essential for cell survival, cell cycle progression and angiogenesis. Amplification/overexpression/activation of PIK3CA (p110α) enzymatic subunit of PI3K and Akt as well as somatic mutation of gene encoding p85α regulatory subunit of PI3K are frequently detected in human ovarian cancer (Shayesteh et al., 1999; Cheng et al., 1992; Yuan et al., 2000; Sun et al., 2001a; Philp et al., 2001). Inhibition of PI3K and/or Akt induces programmed cell death in ovarian cancer cells (Yuan et al., 2000). Expression constitutively active AKT2 results in ovarian cancer cells resistant to cisplatin, taxol, and FTIs-induced apoptosis, whereas dominant negative Akt sensitizes the cells to chemotherapeutic drugs (Herod et al., 1996; Datta et al., 1997). Thus, PI3K/Akt pathway is a critical target for ovarian cancer intervention and inhibition of PI3K and/or Akt could overcome a subset of chemoresistant ovarian cancers. Biological and pharmacological approaches have been tested to inhibit PI3K/Akt pathway.

Biological approaches include antisense, dominant-negative, antibody of PI3K and Akt as well as peptides to mimic and compete pleckstrin-homology (PH) domain of Akt binding to PI3K products, PtdIns-3,4-P2 and PtdIns-3,4,5-P3. We have previously demonstrated that the introduction of antisense AKT2 into several AKT2-overexpressing cancer cell lines abrogated endogenous AKT2 expression and diminished their invasiveness and tumour formation in nude mice (Cheng et al., 1996). Antisense oligonucleotides of Akt can inhibit Akt pathway and induce apoptosis in different cell lines (Liu et al., 2001) and cell growth and survival can also be inhibited by the expression of dominant negative (DN) forms of PI3K and Akt (Sato et al., 2000; Orik et al., 2001). Our recent data show that expression of DN-Akt in NIH3T3 cells remarkably reduces v-H-ras-induced colony formation and tumor formation (unpublished data). Moreover, consistent with the tumor-inhibitory effects of DN-PI3K and DN-Akt is the demonstration of the inhibition of Ras and BCR/ABL malignant transformation with p85ΔiSH2 and DN-Akt, respectively (Rodriguez-Viciana et al., 1997; Skorski et al., 1997). Microinjection of AKT2 antibody into myoblasts can specifically block their function, i.e. induction of myotube (Vandromme et al., 2001). Further studies are required to investigate the effects of antibodies of PI3K and Akt on human cancer cell growth.

The importance of PI3K and Akt in cell survival, growth, cell transformation and human malignancy has prompted the search for specific and safe pharmacological inhibitors for PI3K and Akt. Although wortmanin and LY294002 efficiently abrogate PI3K activity and have been widely used in the cell culture system (Dong et al., 1999; Mills et al., 2001), they have not been applied for clinical trails due to either toxicity (LY294002) or a short of half-life (wortmanin). To date, only one Akt inhibitor is commercially available (Calbiochem). However, there are not enough data to draw the conclusion now. We have recently demonstrated that FTI-277, originally designed to block Ras oncoprotein, inhibits PI3K/Akt pathway and induces apoptosis in a number of human cancer cell lines including ovarian carcinoma (Jiang et al., 2000). ETIs are highly effective at inhibiting tumor growth without toxicity to normal cells. However, the mechanism by which they inhibit tumor growth is not well understood (Cox and Der, 1997; Sebti and Hamilton, 1997; Lebowitz and Prendergast, 1998). FTIs are unable to induce apoptosis in Raf transformed NIH3T3 cells even though MAPK pathway is inhibited by FTIs (Cox and Der, 1997; Sebti and Hamilton, 1997), indicating that ETIs may target other cell survival pathway(s) regulated by Ras or other farnesylated proteins. Interestingly, our data showed that FTI-277 induces apoptosis only in AKT2-overexpressing human cancer cell lines. Furthermore, overexpression of AKT2, but not oncogenic H-Ras, sensitizes NIH3T3 cells to FTI-277; and a high serum level prevents FTI-277-induced apoptosis in H-*Ras*-but not *AKT2*-transformed NIH3T3 cells (Jiang et al., 2000). These data suggest that FTIs specifically target the PI3K/Akt pathway to inhibit tumor cell growth and may be candidate agents for reversing resistance of human ovarian cancer to cisplatin and taxol.

10. Conclusions and future directions

Despite numerous attempts made to improve the therapeutic outcome for ovarian cancer in the past decades, chemoresistance remains a key concern for successful management of this gynecologic malignancy. The advent of molecular and cellular techniques and their applications in research in the regulatory mechanisms of tumor cell fate (i.e. proliferation, differentiation, and apoptosis) has facilitated major advances in the current understanding of the pathobiology of chemoresistance. The recent demonstration that the cell survival intermediates Xiap, PI3K and Akt as key determinants of chemosensitivity in ovarian cancer cells has brought new excitement on their potential role as therapeutic targets and the emergence of new strategies for the treatment of chemoresistant ovarian cancer. It is essential that future work in this area is aimed at developing pharmacological reagents as well as genetic and biochemical approaches that not only identify novel roles for Xiap and Akt but also verify the physiological functions previously ascribed to these intermediates. The generation of a potent and specific Xiap and Akt inhibitors would certainly revolutionize the study of the processes mediated by Xiap and Akt in the same way inhibitors of MAP kinase kinase 1 activation (e.g. PD98059, PD184352, U0126) have on our understanding of processes regulated by the classical MAP kinase pathway. More importantly, such drugs or in combination with conventional chemotherapeutic agents would reasonably improve the outcome of ovarian cancer. Yet, there is still much to be learned about how Akt activity is regulated by Xiap. The mechanism by which

Akt promotes cell survival, especially via postmitochondrial molecules regulated by Akt, is still obscure. Another important question that has not been addressed is whether FTIs, which have been shown to induce apoptosis by inhibition of PI3K/Akt pathway (Jiang et al., 2000), target Xiap and overcome chemoresistance in a subset of ovarian cancer. In addition, while Xiap down-regulation enhances the apoptotic response of chemoresistant ovarian cancer cells induced by adenoviral wild type p53 sense expression in vitro, whether down-regulation of Xiap expression or function could indeed serve as an important adjuvant for *p53* gene therapy, remains to be tested. The inhibition of Xiap function in target cells via expression or delivery of Xiap dominant negatives (e.g. XAF1, DIABLO/Smac and HtrA2/Omi), may prove to be a promising strategy. The realization of these therapeutic potentials will rest upon the success in cell-specific gene targeting and delivery of the candidate "molecule." The outcome of these developments will have profound effects on the management of patients with drug-resistant ovarian cancer.

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Positive Feedback Regulation between Akt2 and MyoD during Muscle Differentiation

CLONING OF *Akt2* PROMOTER*

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Akt2 is a member of the Akt/PKB family, which is involved in a variety of cellular events including cell survival, proliferation, and differentiation. During skeletal muscle differentiation, the Akt2 but not Akt1 expression was significantly increased. Microinjection of anti-Akt2 but not anti-Akt1 antibody efficiently abrogated myogenesis, indicating that Akt2 plays a specific role in muscle differentiation. It has been well documented that ectopic expression of MyoD is sufficient to induce muscle differentiation in myoblasts. However, the mechanism of induction of Akt2 during muscle differentiation and the significance of Akt2 protein in MyoD-induced myogenesis are largely unknown. In this study, we provide direct evidence that *Akt2* is transcriptionally regulated by MyoD and activates MyoD-myocyte enhancer binding factor-2 (MEF2) transactivation activity. The *Akt2* promoter was isolated and found to contain nine putative E-boxes (CANNTG), which are putative MyoD binding sites. Electrophoretic mobility shift analyses revealed that MyoD bound to eight of the sites. The expression of MyoD significantly enhanced *Akt2* promoter activity and up-regulated Akt2 mRNA and protein levels. Moreover, Akt2 but not Akt1 was activated during differentiation. The expression of Akt2 activated MyoD-MEF2 transcriptional activity and induced myogenin expression. These data indicate that there is a positive feedback regulation loop between Akt2 and MyoD-MEF2 during muscle differentiation, which is essential for MyoD-induced myogenesis.

Skeletal muscle differentiation requires an ordered multiple step process in which myoblasts irreversibly exit from the cell cycle, elongate, and fuse into multinucleated myotubes. This program is driven by the expression of the MyoD family of transcription factors and the myocyte enhancer binding factor-2 (MEF2)[1] family members (1). The MyoD family (also called myogenic regulatory factors) of basic helix-loop-helix proteins includes MyoD, myogenin, Myf5, and myogenic regulatory factor-4. Forced expression of MyoD transcription factor can inhibit cell cycle progression and induce muscle differentiation. The transcription of many muscle-specific genes is activated by the binding of MyoD factors to a simple consensus sequence of CANNTG termed an E-box, present in regulatory regions of many muscle-specific genes. The MEF2 family of transcription factors includes MEF2A, MEF2B, MEF2C, and MEF2D, which belongs to the MADS (MCM1, agamous, deficiens, serum response factor) box transcription factors. Evidence indicates that the members of MyoD and MEF2 families interact with each other to synergistically induce muscle-restricted target genes (2). One of the targets is the gene encoding myogenin, which is one of the earliest molecular markers for myoblasts committed to differentiation. The up-regulation of myogenin in concomitant with the induction of the cyclin-dependent protein kinase inhibitor $p21^{Waf/Cip1}$ indicates that the cells have irreversibly exited from the cell cycle and entered the differentiation program (3).

Unlike most growth factors that stimulate myoblast proliferation and inhibit muscle differentiation, the insulin-like growth factors (IGF-I and IGF-II) are potent stimulators of muscle differentiation through induction of myogenin and MEF2 (4). However, the intracellular myogenic signaling process dependent on IGFs is poorly understood. Studies on signaling through IGF receptors have revealed two main pathways, MAPK and PI3K, by which these signals might be transmitted. Several reports showed that the PI3K inhibitors (LY294002 and wortmannin) and a dominant negative p85α (the PI3K regulatory subunit) block IGF-induced myogenesis, whereas the MAPK inhibitor PD098059 enhanced IGF-stimulated muscle differentiation (5–7). Moreover, recent studies demonstrate that constitutively activated PI3K enhanced the transcriptional activity of both MyoD and MEF2 (6, 8). Taken collectively, these studies strongly indicate the essential role of PI3K in myogenesis.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF452411.

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[1] The abbreviations used are: MEF2, myocyte enhancer binding factor-2; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; HEK, human embryonic kidney; Luc, luciferase; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP-response element-binding protein; HDAC, histone deacetylase; PI3K, phosphatidylinositol 3-kinase; IGF, insulin-like growth factor.

The serine/threonine protein kinase Akt (also named PKB) is a major downstream target of PI3K and has been implicated in muscle differentiation (9). Three different isoforms of Akt have been identified including Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, all of which are activated by growth factors in a PI3K-dependent manner. The full activation of the Akt requires phosphorylation at Thr^{308} (Akt1), Thr^{309} (Akt2), or Thr^{305} (Akt3) in the activation loop and Ser^{473} (Akt1), Ser^{474} (Akt2), or Ser^{472} (Akt3) in the C-terminal activation domain (10). The most studied isoform is Akt1, which mediates IGF signaling to regulate cell survival, cell growth, GLUT4 translocation, and muscle differentiation. It has been shown that ectopic expression of constitutively activated Akt1 can promote extensive differentiation in different myoblast cell lines in the absence of IGF-I and can reverse the inhibitory effects of PI3K inhibitors LY294002 and wortmannin on myogenic differentiation (5, 9, 11). However, several studies including ours show that both the mRNA and protein levels of the endogenous Akt1 were not changed, whereas Akt2 was elevated during muscle differentiation, suggesting that Akt2 but not Akt1 plays a specific role in myogenesis under physiological condition (12–14). A recent study provides compelling supporting evidence by showing that microinjection of Akt2 antibody inhibited the differentiation of muscle cells, whereas anti-Akt1 antibody did not inhibit cell differentiation (15). However, the mechanism by which Akt2 is involved in myogenesis is currently unknown. In this study, we cloned the *Akt2* promoter and demonstrated that MyoD transcriptionally regulates AKT2. During muscle differentiation, elevated Akt2 in turn activated MyoD-MEF2 transactivation activity resulting in myogenin expression.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Materials—Human epithelial kidney (HEK)293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. C2C12 mouse myoblasts were grown in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (growth medium). To induce differentiation, C2C12 cells were maintained in Dulbecco's modified Eagle's medium containing 10% horse serum (differentiation medium). pCSA-MyoD was kindly provided by Dr. Lassar (Harvard Medical School, Boston, MA). The FLAG-tagged MyoD was constructed by subcloning MyoD into p3X-FLAG-CMV10 (Sigma). MEF2 and myogenin-Luc plasmids are described elsewhere (6). The antibodies to Akt1 and Akt2 were purchased from Upstate Biotechnology, and anti- MyoD, myogenin, actin, and FLAG antibodies were from Santa Cruz Biotechnology.

Transcription Start Site Mapping of Human AKT2 Gene—For the analysis of the Akt2 transcription start site, human OVCAR3 mRNA was reverse-transcribed at 55 °C using Superscript reverse transcriptase (Invitrogen) and an Akt2 exon 1-specific reverse complement oligonucleotide 5′-TTCTTTGATGACAGACACCTCATT-3′. Synthesized cDNAs were amplified by polymerase chain reaction using a series of forward primers specific for the DNA sequences within the 8,500 bp upstream of the translation start site and a reverse primer from the coding region of exon 1 (GenBank™ accession number AF452411), and the products of these reactions were resolved by agarose gel electrophoresis.

Cloning and Analysis of the Human Akt2 Promoter—For the reporter analysis of the *Akt2* promoter, DNA fragments containing *Akt2* genomic sequences were amplified from a cosmid clone (pWE9–3), which was obtained by the screening of a human placenta genomic library (Stratagene) with 5′ sequence of *Akt2* cDNA using the polymerase chain reaction and primers derived from human genomic *Akt2* (GenBank™ accession number NT011250). Amplified DNA fragments were subcloned into the luciferase reporter vector pGL3 (Promega). The integrity of all constructs was confirmed by DNA sequencing. Luciferase assays were performed using the luciferase assay system (Promega), and activities were normalized to β-galactosidase activity.

Northern and Western Blot Analysis—Northern blot analysis of total cellular RNA was performed according to standard procedures. Hybridized ^{32}P-labeled probes were visualized and quantified using PhosphorImager analysis (Molecular Dynamics). Western blot analysis was performed as described previously (12).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Analysis—The nuclear extracts were prepared from the FLAG-tagged MyoD-transfected HEK293 cells following the procedures described previously (16). Normalized extracts containing 3–8 μg of protein were incubated with ^{32}P-radiolabeled double-stranded oligonucleotide probes containing each of the nine MyoD binding sequences: 5′-GTCTCAAGTGAGGCAGGTGGTGAGCCCATAA-3′ (−1861/−1832); 5′-GTGCAAGGGGTTCCACCTGCTGCCCCTCCC-3′ (−1750/−1722); 5′-GCCCCGGCTCTCTCACCTGCTTCCGGTTTTG-3′ (−1512/−1483); 5′-GAGGCAAGTGCTGTGAGGGGAAGGGAACGCT-3′ (−982/−953); 5′-GCGGGGCCAAGGCAGATGGCCCCTGGCAGC-3′ (−754/−724); 5′-GCCTCCAGCCCCACCTGCTCCAAGCAGA-3′ (−432/−403); 5′-GCTCCAAGCAGACAGATGGGGCGAGTGTAG-3′ (−415/−386); 5′-GTAGGTAAAAATTCACCTGTTCAGAAACATA-3′ (−260/−231); and 5′-GTAGCTGGAATTACAGGTGCCTGCCACCAG-3′ (−56/−22). Protein-DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Competition and Supershift Controls for Electrophoretic Mobility Shift Analysis—For competition controls, nuclear extracts were incubated with radiolabeled probes in the presence of 100-fold molar excess unlabeled competitor probe prior to PAGE. For supershift assay, 1 μl of anti-FLAG antibody was incubated with nuclear extracts for 20 min at room temperature prior to the addition of radiolabeled probe and PAGE.

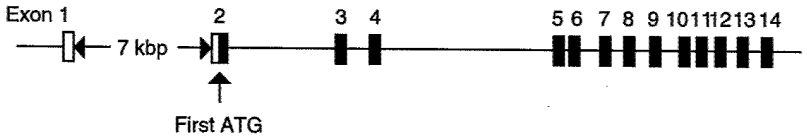
RESULTS

Akt2 Promoter Contains Nine MyoD Binding Elements—To analyze the transcriptional regulation of the serine/threonine protein kinase *Akt2*, we cloned the 5′-flanking region of Akt2 gene from a pWE-15 cosmid human placenta genomic library using the 5′-non-coding region of *Akt2* cDNA as probe. Three overlapping cosmid clones were obtained. Sequence analyses revealed that the Akt2 gene consists of 14 exons. Exon 1 is an untranslated region, and the first intron is >7.6 kilobases in length. The translation initiation site, ATG, of Akt2 protein resides within the exon 2 (Fig. 1*A*). The transcription start site, which was determined by 5′-RACE PCR, lies 7,829 bp upstream of the translation start site. Transcription element analyses of the 2,000 bases of upstream of the transcription start site of the *Akt2* gene, which is considered the putative Akt2 promoter, revealed multiple binding sites for MyoD, Oct1, and p300 and single sites for AP1, C/EBPβ, C/EBP, CREB, and SP1 (Fig. 1*B*). The transcription factor that has the most binding sites in Akt2 promoter is MyoD (nine putative MyoD binding sites: −1852/−1841, −1741/−1731, −1502/−1493, −981/−972, −759/−736, −422/−413, −405/−396, −250/−242, and −41/−32). A MyoD binding site is also called an E-box and its consensus sequence is CANNTG (Fig. 1*B*).

Defining the MyoD Binding Site(s) in the Akt2 Promoter—To determine the MyoD binding elements, we carried out the electrophoretic mobility shift analysis. Nine double-stranded oligonucleotides, each containing an E-box from the *Akt2* promoter, were labeled with ^{32}P and incubated with the nuclear extract from FLAG-tagged MyoD-transfected HEK293 cells. The quality of the nuclear extracts was examined with oligonucleotides derived from an E-box of MEF-2 (data not shown). Mobility shift was observed in −1861/−1832, −1750/−1722, −1512/−1483, −754/−726, −432/−403, and −415/−386, −260/−231, and −51/−22 fragments (Fig. 1*C*). The formation of the electrophoretically retarded complexes was inhibited when an excess of unlabeled oligonucleotides (competitor) were introduced (*middle lane* of each E-box). Moreover, an addition of an anti-FLAG antibody to the reaction mixtures induced the supershift of the protein-DNA complexes appearing in −1861/−1832, −1750/−1722, −1512/−1483, −754/−726, −432/−403, −415/−386, −260/−231, and −51/−22 (Fig. 1*C*). These results indicate that eight of the *Akt2* protomer-derived E-box oligonucleotides can specifically bind MyoD.

MyoD Transactivates the Akt2 Promoter—To investigate whether MyoD regulates the transcription of *Akt2*, a 3.1-kilobase genomic fragment corresponding to the region from bases −2898 to +220 containing nine putative MyoD binding sites,

A



B

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MyoD, MyoD, MyoD, Oct1, p300, AP1, Oct1, MyoD, Sp1, MyoD, CREB, CCAAT, C/EBP, MyoD, MyoD, p300, MyoD, Oct1, C/EBP, MyoD

C

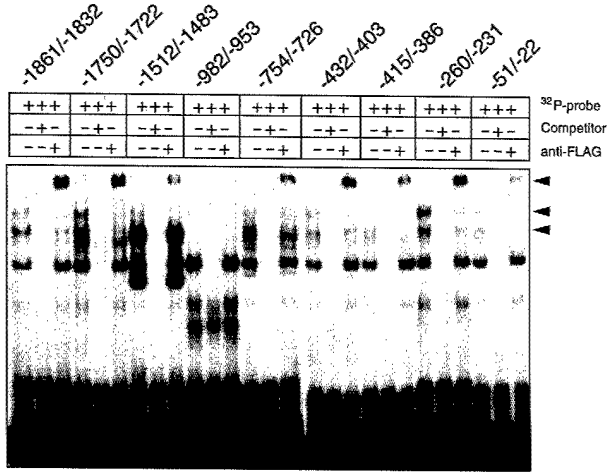

FIG. 1. **Human *Akt2* promoter contains multiple MyoD binding sites.** *A*, schematic representation of the human *Akt2* genomic locus. The exons are shown as *boxes 1–14*. *B*, *Akt2* promoter sequence. Putative transcription factor binding sites are *boxed*. *C*, MyoD binding to the DNA element from the *Akt2* promoter. The electrophoretic mobility shift analysis of double-stranded oligonucleotides containing each MyoD binding sites as indicated at the top. Equal amounts of ^{32}P-labeled oligonucleotides were incubated with nuclear extract prepared from FLAG-MyoD-transfected HEK293 cells in the presence or absence of a 100-molar excess of the unlabeled oligonucleotides (competitor). Supershift was examined by incubation of the reactions with anti-FLAG antibody.

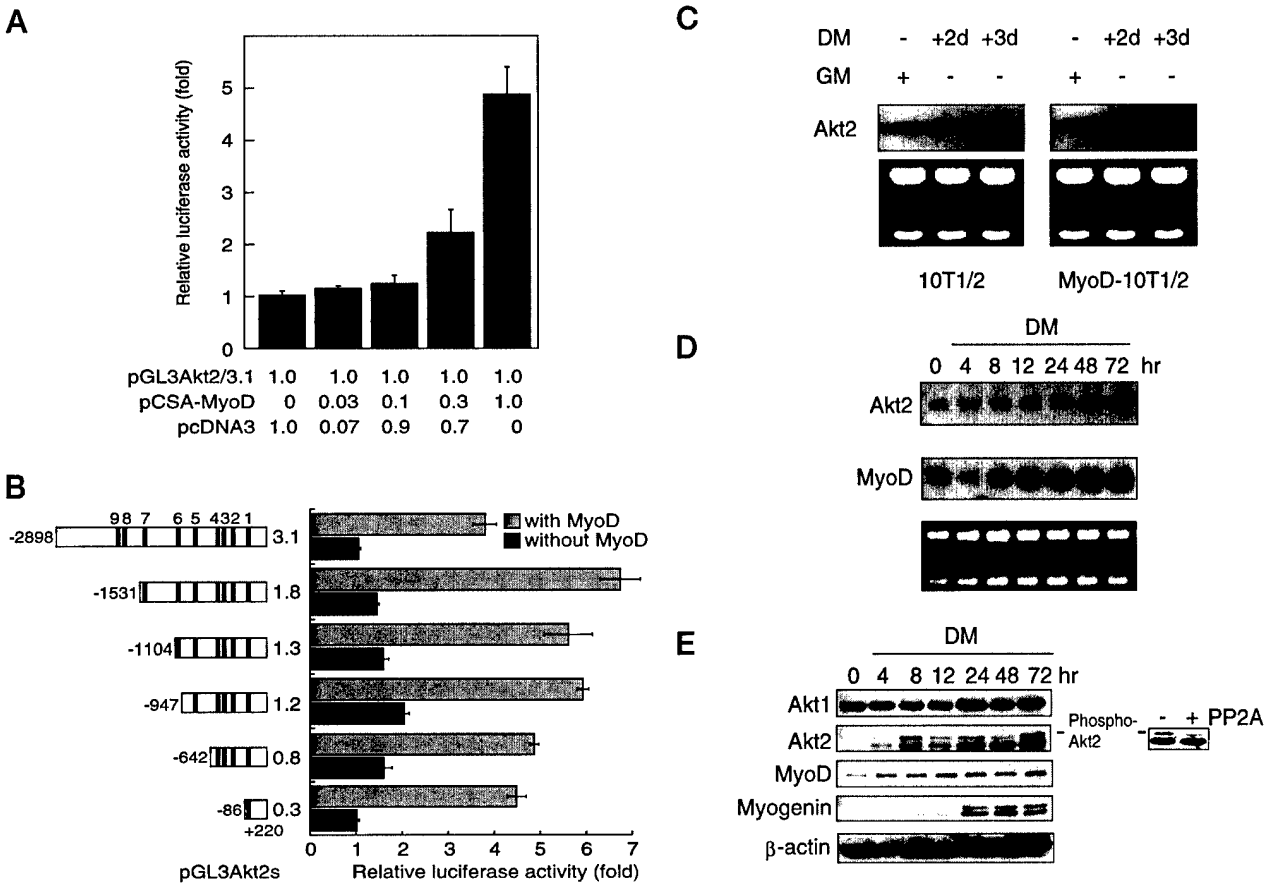

FIG. 2. **MyoD transactivates the *Akt2* promoter and induces Akt2 expression.** *A*, MyoD-induced Akt2 promoter activity occurs in a dose-dependent manner. HEK293 cells were transfected with different amounts of MyoD and pGL3Akt2/3.1 reporter, which contains all nine E-boxes in the presence of tracer amounts of β-galactosidase. Luciferase activity in cell lysates at 36 h posttransfection was normalized to galactosidase and expressed relative to values obtained using the empty reporter plasmid. Results shown are the mean ± S.E. of three independent experiments performed in triplicate. *B*, the first MyoD binding site (−51/−22) is sufficient to activate the Akt2 promoter. HEK293 cells were transfected with the indicated different lengths of Akt2 reporters together with or without MyoD. *C*, Akt2 mRNA is induced in MyoD-transfected but not parental 10T1/2 cells. The cells were cultured in growth medium (*GM*) for 24 h (1 day) and then switched to differentiation medium for 48 (*+2d*) and 72 h (*+3d*). Total RNAs (20 μg) were subjected to Northern blot analysis with [^{32}P]dCTP-labeled Akt2 probe. *D* and *E*, Akt2 is elevated at mRNA and protein levels and activated during C2C12 cell differentiation. The C2C12 cells were cultured in differentiation medium (growth medium) for the indicated times. RNA and cell lysates were analyzed by Northern and Western blots with indicated isotope-labeled probes and antibodies, respectively. Phosphorylated Akt2 migrated slower and was abrogated by PP2A phosphatase treatment (*E*, panel 2).

was subcloned upstream of the luciferase gene in pGL3 basic vector (pGL3-AKT2/3.1). A co-transfection of pGL3-Akt2/3.1 with MyoD into HEK293 cells resulted in a significant increase in reporter activity compared with the control sample co-transfected with the reporter and an empty vector (pcDNA3). Moreover, *Akt2* promoter is regulated by MyoD in a dose-dependent manner (Fig. 2*A*). A similar level of induction of Akt2 reporter activity was also observed upon the transfection of 10T1/2 cells, which lack the endogenous MyoD (data not shown).

To define the MyoD-responsive regions in this promoter, we constructed a group of deletion reporters containing the Akt2 promoter serially deleted from the 5′ end of the −2898 to +220 fragment (Fig. 2*B*). The deletion of −2898 to −1531 significantly increased the MyoD responsiveness by ~40%, even though two potential myoD binding sites were eliminated, suggesting the presence of inhibitory elements for MyoD responsiveness within this region. The further deletion of E-box 7 reduced MyoD responsiveness by 15%. The deletion of the region from −642 to −86 (pGL3-AKT2–0.3), containing a cluster of three E-boxes, deceased the MyoD responsiveness by ~16%. Nevertheless, pGL3-AKT2–0.3, which contains only an E-box, was still induced by MyoD >4.5-fold (Fig. 2*B*), suggesting that the E-box 1 could be a major MyoD response site within the promoter.

Akt2 Is Induced by MyoD during the Muscle Differentiation—We next examined whether MyoD induces mRNA of *Akt2*. Because 10T1/2 myoblast do not express MyoD and are unable to differentiate to myotubes, we have established a 10T1/2 cell line, which was stably transformed with a *MyoD* expression vector. These *MyoD*-transformed cells expressing myocyte-specific markers form multinucleated myotubes when exposed to mitogen-poor differentiation medium (17, 18). The levels of *Akt2* mRNA were evaluated in parental and MyoD-transfected 10T1/2 cells in both growth medium and differentiation medium. *Akt2* mRNA was significantly increased in the 10T1/2-MyoD cells, but this induction did not occur in the parental 10T1/2 fibroblasts when exposed to the differentiation culture medium (Fig. 2*C*).

We further investigated the status of Akt1 and Akt2 in C2C12 cells, which express endogenous MyoD, during differentiation. Western immunoblot analysis revealed that Akt1 protein is stably expressed at a relative high level prior to and during differentiation (Fig. 2*E*). However, both mRNA and protein levels of Akt2 were very low in C2C12 myoblasts cultured in high mitogen growth medium but progressively increased following exposure of cultures to differentiation medium (Fig. 2, *D* and *E*). Moreover, Akt2 kinase activity was induced after switching the culture to differentiation medium

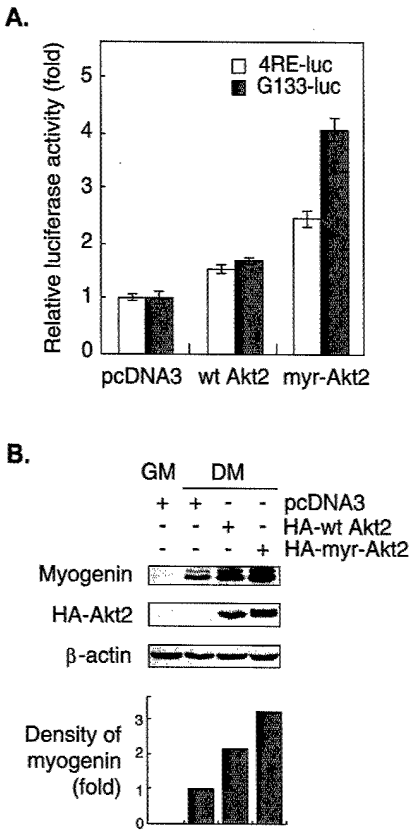

FIG. 3. **Akt2 activates the MyoD and MEF2 transactivation activities and induces myogenin expression.** *A*, wild type and constitutively active Akt2 induce MyoD- and MEF2-dependent reporter activity. C2C12 cells were transfected with different forms of Akt2 along with either 4RE-Luc, which contains four MyoD binding sites, or G133-Luc having both MEF2 and MyoD binding sites. After 24 h of the transfection and 16 h of incubation in differentiation medium, cell lysates were subjected to luciferase assay analysis. *B*, C2C12 cells were transfected with indicated plasmids, cultured in growth medium for 24 h, and then replaced with differentiation medium for 16 h. The expression of myogenin and transfected Akt2 was analyzed by Western blot analysis.

(Fig. 2*E*), suggesting an important role for Akt2 in myogenesis.

Akt2 Induces Myogenin and Activates MyoD-dependent Reporter Genes—The induction of mRNA/protein and kinase activity of Akt2 during muscle differentiation suggests that it regulates muscle-specific gene(s) that controls differentiation. In fact, a previous report shows that ectopical expression of Akt1 and Akt2 could induce muscle-specific gene muscle creatine kinase, and Akt2 was more effective than Akt1 (15). However, the mechanism of Akt induction of muscle-specific gene expression has not been well documented. To explore this hypothesis further, we tested the effects of Akt2 on myogenin expression and MyoD transcriptional activity. G133-Luc, which is a 133-bp myogenin proximal promoter containing MyoD and MEF2 binding sites or 4RE-Luc, which is a MyoD-dependent reporter gene containing four MyoD binding sites, was co-transfected into C2C12 myoblasts with either wild type Akt2 or constitutively active Akt2. The expression of the wild type Akt2 induced G133-Luc and 4RE-Luc reporter activities at 1.6- and 1.5-folds, respectively, whereas the levels of G133-Luc and 4RE-Luc reporter activities were significantly increased (4.2- and 2.5-fold) in the cells transfected with the constitutively active Akt2 (Fig. 3*A*). Consistent with the reporter results, myogenin expression was induced by both wild type and constitutively active Akt2 in C2C12 cells after 16 h of exposure to differentiation medium (Fig. 3*B*). These data suggest that

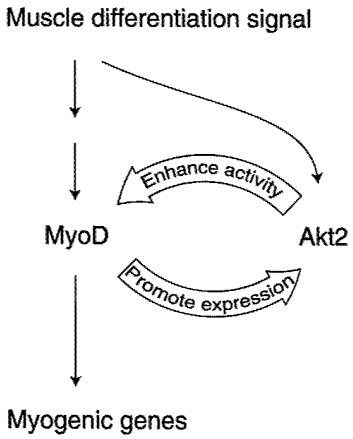

FIG. 4. **Schematic illustration of positive feedback regulation between Akt2 and MyoD during the muscle differentiation.**

AKT2 can up-regulate the endogenous myogenin expression and promote the MyoD transcriptional activity during muscle differentiation.

DISCUSSION

Previous studies demonstrate that IGF1-induced muscle differentiation was primarily mediated by the PI3K/Akt pathway (5–9). Among the three isoforms of Akt family, Akt2 is highly expressed in skeletal muscle (12) and plays a specific role in muscle differentiation as demonstrated by up-regulation of Akt2 but not Akt1 and Akt3 during differentiation and abrogation of myotube formation by anti-Akt2 antibody (15). However, the mechanisms by which Akt2 is up-regulated during the muscle differentiation and stimulates myotube formation are currently unclear. In this report, we provide evidence showing that Akt2 promoter possesses multiple MyoD binding sites, that the expression of Akt2 was induced by MyoD through stimulation of its promoter activity, and that the elevated Akt2 activated MyoD transactivation and induced muscle-specific gene myogenin expression to trigger muscle cell differentiation. Our data indicate a positive feedback regulation loop between Akt2 and MyoD during skeletal muscle differentiation (Fig. 4).

In ectopic expression systems, three isoforms of Akt display very similar functions including muscle differentiation. In fact, previous studies have mostly focused on Akt1 and demonstrated that Akt1 is a critical intermediate in IGF1-induced muscle differentiation hypertrophy and muscle survival (5–9, 19). A previous study shows that the expression of constitutively activated Akt1 induces the transactivation activity of MyoD and MEF-2 (6). However, under physiological conditions, Akt2 seems to play more important roles in myogenesis, because mRNA and protein levels of Akt2 but not Akt1 were up-regulated during muscle differentiation (Fig. 2). In addition, accumulated studies have shown clear differences between these three isoforms in terms of biological function. (*a*) *Akt1* expression is relatively uniform in various normal organs, whereas high levels of *Akt2* are detected in skeletal muscle and heart (12, 20, 21). (*b*) The inhibition of Akt2 but not Akt1 expression abrogates IGF1-induced muscle differentiation (15). (*c*) *Akt2* and *Akt3* but not *Akt1* are amplified and/or up-regulated in certain types of human cancer (22). (*d*) NIH3T3 cells are transformed by wild type Akt2 but not Akt1 and Akt3 (23). (*e*) *Akt2*- and *Akt1*-deficient mice displayed different phenotypes. *Akt2* knock-out mice exhibited a typical type 2 diabetic phenotype that cannot be compensated by the presence of *Akt1* and *Akt3* (24). In contrast, $Akt1^{-/-}$ mice exhibited no diabetic phenotype (25, 26) but showed an impairment in organismal growth, *i.e.* smaller when compared with wild type littermates.

Such relatively subtle phenotype of Akt1$^{-/-}$ mice suggests that Akt2 and Akt3 may substitute to some extent for Akt1 (25). Nevertheless, these data indicate that there are non-redundant functions between three isoforms of Akt in certain tissue and/or cell types. In this study, we cloned *Akt2* promoter and identified multiple MyoD binding sites in *Akt2* (Fig. 1) but not in *Akt1* promoter,[2] further indicating the different transcriptional regulation between *Akt1* and *Akt2*.

Previous studies have shown that MyoD and MEF2 transcription factors interact with each other to synergistically induce muscle-restricted target gene expression resulting in muscle differentiation (2), and that class II histone deacetylase (HDAC) 4 and 5 bind to MEF2 and inhibit MEF2/MyoD transactivation activity and muscle differentiation (27). Calcium/calmodulin-dependent protein kinase induces muscle differentiation by phosphorylation of HDACs 4 and 5 and shuttling of the phosphorylated HDACs from nuclear MEF2·HDAC complex to the cytoplasm (28). It has been also shown that class II HDACs-repressed muscle differentiation can be overcome by treating cells with IGF1, which induces HDACs export from the nucleus (29). However, the mechanism by which IGF1 regulates HDACs 4 and 5 has not been well characterized. In this report, we have shown that the protein level and kinase activity of Akt2 were elevated during muscle differentiation. The up-regulation of Akt2 was because of MyoD induction of Akt2 promoter activity, whereas activated Akt2 might result from autocrine production of IGFs by myoblasts under differentiation medium condition (4). Nevertheless, elevated Akt2 during muscle differentiation could mediate IGFs signals to regulate HDACs 4 and 5 functions, even though a previous study showed that Akt1 did not phosphorylate HDACs 4 and 5 (28). Additional studies are required to define the mechanism of Akt2 activation of MyoD-MEF2 transcriptional activity.

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Telomerase Is Regulated by c-Jun NH_2-Terminal Kinase in Ovarian Surface Epithelial Cells[1]

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Abstract

Telomerase activity is present in >90% of all tumors and appears to be regulated by the phosphatidylinositol 3-kinase signaling pathway. Here we demonstrate that Akt is not involved in the signaling cascade for telomerase regulation in ovarian surface epithelial cells. However, we showed that c-Jun NH_2-kinase induces telomerase activity, that inhibition of JNK by JIP abrogates telomerase activity, and that JNK expression activates transcription of a reporter gene fused to the hTERT promoter sequence. Consequently, our data show that JNK is a key regulator of telomerase activity and, hence, may provide new perspectives on tumorigenesis that could be exploited for novel therapeutic strategies.

Introduction

Because telomerase is present in >90% of human tumors and is absent from most normal somatic cells (1), it is the most widely expressed and specific cancer marker presently known (2). Consequently, understanding the molecular mechanisms regulating telomerase is of significant clinical importance. Transcriptional control of telomerase includes alternate splicing of hTERT[3] mRNA transcripts (3), hTERT promoter methylation (4), and binding of one or more transcription factors, including c-Myc, to the hTERT promoter (5). Protein kinase C (6), DMSO (7), calcium (8), and zinc (9) are also reported to affect telomerase activity, illustrating the complexity associated with telomerase regulation. We and others have shown previously that PI 3-kinase can up-regulate telomerase activity (10, 11). In the present study, we show that the primary target of PI 3-kinase, Akt, did not affect telomerase activity in human OSE cells. However, JNK, also a downstream target of PI 3-kinase, increased telomerase activity, induced hTERT transcription, and led to induced reporter activity by transcriptional activation of hTERT. Therefore, this is the first report to identify JNK as a key regulator of telomerase activity.

Methods and Materials

Cell Culture. Telomerase-positive ovarian carcinoma cell lines OV1063, OV2008, OVCAR3, SW626, and CaOV3 were used. Four nontumorigenic SV40 large-T antigen-transfected OSE cell lines, FHIOSE 118, FHIOSE 1816–686, NFHIOSE 80, and FHIOSE 1816–575, derived from normal ovarian surface epithelium (12), were also used in this study. FHIOSE 118, NFHIOSE 80, and FHIOSE 1816–575 cells were determined previously to be telomerase negative (13). Cells were maintained in Medium 199/MDCB 105 (1:1; Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) and 10 μg/ml gentamicin (Life Technologies, Inc., Grand Island, NY) in a humidified 5% CO_2/95% air atmosphere. Cell growth was determined by MTS assay as described previously (8).

Treatment with Anisomycin. To determine whether JNK signaling was involved in telomerase regulation, SW626 or FHIOSE 118 cells were serum starved for 24 h, treated for 8 or 24 h with 20 or 40 μM of anisomycin (Sigma), a potent stimulator of JNK, and then assayed for telomerase activity. Parallel cultures of cells were collected for RT-PCR.

Transfections. To examine the role of JNK signaling in telomerase regulation, Flag-tagged JNK, Flag-tagged JIP, which is a specific inhibitor of JNK, HA-tagged AA, and GFP were used for transfection. Cells were serum starved for 24 h and then transfected with 3 μg of DNA using Lipofectamine reagent (Life Technologies, Inc., Grand Island, NY). Cells were collected at 24 h after transfection and assayed for telomerase activity. In all transfection experiments, parallel cultures transfected with GFP were used as a control for transfection efficiency.

Telomerase Assay. To quantitatively detect changes in telomerase levels, all cells were assayed for telomerase activity using the telomerase PCR-ELISA (Roche Molecular Biochemicals, Indianapolis, IN), as described previously (13) and according to the manufacturer's instructions. After PCR-ELISA, telomerase activity was detected using a Dynex-MRX plate reader (Dynex Technologies, Chantilly, VA) and recorded as absorbance units. These values were expressed as a fold increase above control levels, with the control value used as the denominator for the determination of fold increase for the treated samples. For graphical representation of the effect of Akt, anisomycin, JNK, or JIP on telomerase activity, control values for the untreated samples of all cell lines were set at 1.0. Telomerase activity is shown ±SE.

Immunoprecipitation and Western Blot Analysis. Cells were lysed on ice using a modified RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.5% NP40, 5 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 1 mM benzamidine, and 10 μg/ml trypsin inhibitor]. Lysates were then centrifuged at 12,000 × at 4°C for 10 min, and the protein concentrations of the supernatants were determined as described previously (10). Protein extracts were solubilized in SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol). Samples containing equal amounts of protein (25 μg) were separated on a 10% SDS-PAGE and electroblotted onto Hybond-ECL nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ) by wet transfer. Immunoblotting was performed using antibodies against JNK (1:1000), phospho-JNK (1:1000), phospho-c-Jun (1:1000), phospho-SEK1 (1:2000), Akt (1:1000), phospho-Akt (1:1000; Cell Signaling, Beverly, MA), β-actin (Sigma; 1:5000), HA (1:1000), and Flag (1:1000). Blots were visualized using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

RT-PCR. To examine the contribution of transcriptional control in telomerase regulation, RT-PCR was performed as described previously (10). To insure there was no DNA contamination, each sample for reverse transcription was prepared in duplicate, with the duplicate preparation lacking reverse transcriptase (14). The cDNA samples were amplified using the Perkin-Elmer GeneAmp kit. The hTERT primers used were hTERT-S (CGGAAGAGTGTCTGGAGCCAA) and hTERT-AS (GGATGAAGCGGAG TCTGGA) oligonucleotides (Sigma Genosys, The Woodlands, TX) with β-actin primers Actin-S (CAGGTCATCACCATTGGCAATGAGC) and Actin-AS (GATGTCCACGT CACACTTCATGA) for an internal control. The amplified products were then separated by electrophoresis on a 9% polyacrylamide gel, stained with 1× SyberGreen (FMC Bioproducts, Rockland, ME), and analyzed

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[3] The abbreviations used are: hTERT, human telomerase reverse transcriptase; PI 3-kinase, phosphatidylinositol 3-kinase; OSE, ovarian surface epithelial; JNK, c-Jun NH_2-terminal kinase; RT-PCR, reverse transcription-PCR; HA, hemagglutinin; AA, activated Akt; GFP, green fluorescent protein.

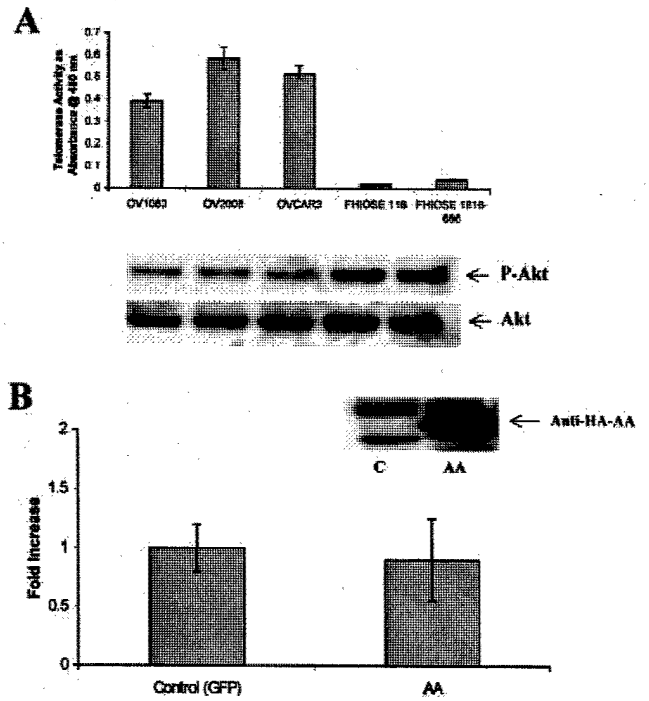

Fig. 1. Akt does not regulate telomerase activity in OSE cells. ovarian cancer cell lines and nontumorigenic OSE cell lines were assayed for telomerase activity. In parallel cultures, phospho-Akt levels were examined using Western blot analysis. Akt levels are indicated as a loading control. FHIOSE 118 cells were transfected with HA-tagged AA and GFP as a control. Cells were collected at 24 h and then assayed for telomerase activity. Successful transfection was confirmed by Western blot analysis. For all samples, telomerase levels are expressed as fold increase; SE.

with the Kodak EDAS 120 Digital Analysis System. Net hTERT mRNA intensities from treated samples were normalized to their corresponding β-actin mRNA levels and were expressed as a percentage of the control samples that were similarly normalized to their corresponding β-actin mRNA levels.

Luciferase Assay. The hTERT promoter-luciferase construct called pGL3-1375 (15) was used to measure hTERT transcriptional activity in the FHIOSE 118 cell line. pGL3-1375 contains a 1375-bp promoter fragment of hTERT fused to the luciferase reporter gene in pBluescript (Stratagene, La Jolla, CA). Transient cotransfections were performed as mentioned above. FHIOSE 118 cells were transfected using Lipofectamine (Life Sciences Technologies) with the indicated amounts of JNK or JIP cDNA. The total amount of transfected DNA was kept constant in each experiment by adding vector-only plasmid. A plasmid expressing the bacterial β gene was also cotransfected in each experiment to serve as an internal control for transfection efficiency. Cells were collected at 48 h, and transcriptional activity was measured as a function of luciferase activity using the Luciferase Assay System (Promega Corp., Madison, WI) according to the manufacturer's instructions and as described previously (16). At the time of collection, cells were observed microscopically to ensure that cells were viable and that there were no signs of apoptosis. Transcriptional activity was expressed as relative luciferase activity $\pm$ SE, after normalization with β activity. Each transfection was performed in triplicate.

Statistical Analysis. Samples for telomerase PCR-ELISA and luciferase assay were run in triplicate, and the data were subjected to the Student's test analysis for determination of statistical significance.

Results

Akt Is Not Involved in Telomerase Regulation in OSE Cells. To determine whether Akt, the primary target of PI 3-kinase, was the downstream target of PI 3-kinase in the regulation of telomerase, several telomerase-positive cancer cell lines and telomerase-negative normal cell lines were surveyed for telomerase activity (Fig. 1). Parallel cultures were assayed by immunoprecipitation and Western immunoblotting for phospho-Akt as well as Akt. Working within the linear rage for AKT phosphorylation, we found an inverse correlation between telomerase activity and Akt activation. Telomerase-negative cell lines demonstrated higher levels of phosphorylated Akt than the telomerase-positive cell lines. Densitometric analysis confirmed this finding. When normalized to total Akt levels, endogenous phosphorylated Akt levels in telomerase-negative cells were 1.8-fold greater than in telomerase-positive cells, as determined by using ImageQuant software. In addition, transfection of activated AA into the telomerase-negative FHIOSE 118 cells did not induce telomerase activity (Fig. 1). Western blot analysis using anti-HA confirmed successful transfection of AA.

Anisomycin Induces Telomerase Activity. Because JNK is also a downstream target of PI 3-kinase, SW626 and FHIOSE 118 cells were treated with anisomycin, a known stimulator of JNK, and then assayed for telomerase activity (Fig. 2). Treatment with anisomycin resulted in a 3- and 3.5-fold increase in telomerase activity in SW626 cells by 8 and 24 h, respectively (Fig. 2). After anisomycin treatment, RT-PCR revealed transcription of hTERT mRNA in FHIOSE 118

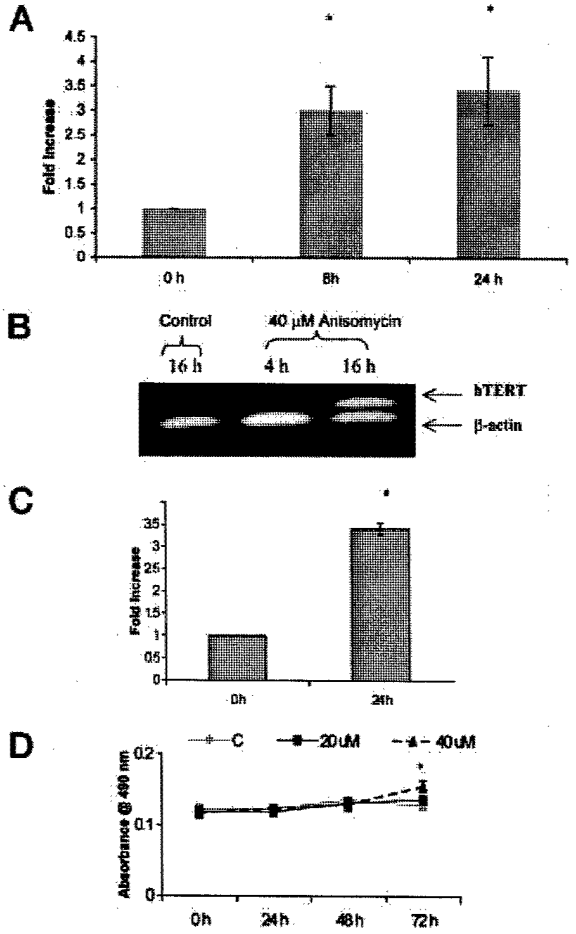

Fig. 2. Anisomycin induces telomerase activity as well as transcription of hTERT. telomerase-positive SW626 cells were treated with 40 μM anisomycin. Cells were collected at 8 and 24 h and then assayed for telomerase activity. *, ≤ 0.02. parallel cultures of FHIOSE 118 cells were treated with 40 μM anisomycin, and RNA was collected at 4 and 16 h, before maximal induction of telomerase activity. RT-PCR revealed transcription of the hTERT component by 16 h. β-actin was used as a control. telomerase-negative FHIOSE 118 cells were treated with 40 μM anisomycin, collected at 24 h, and assayed for telomerase activity. *, ≤ 0.006. Telomerase levels are expressed as fold increase; SE. anisomycin treatment did not affect cell viability as determined by a cell proliferation assay (Fig. 2). *, $= 0.74$.

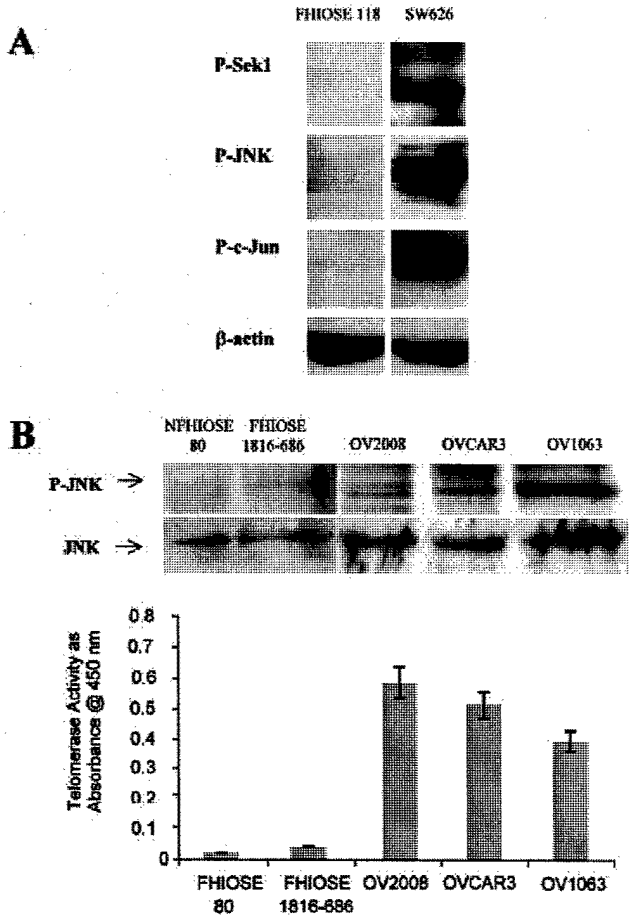

Fig. 3. Endogenous phospho-JNK is elevated in telomerase-positive ovarian cancer cells. FHIOSE 118 and SW626 cells were collected and lysed as described previously. Using Western blot analysis, membranes were probed with phospho-SEK1, phospho-JNK, and phospho-c-Jun. β-actin was used as a loading control. telomerase-positive and -negative cells were examined for endogenous levels of phospho-JNK using Western blot analysis. JNK was used as a loading control. Parallel cultures were examined for telomerase levels.

cells, indicating that JNK can both increase endogenous telomerase activity in telomerase-positive cells as well as induce telomerase activity in telomerase-negative FHIOSE 118 cells (Fig. 2). Similarly, treatment with anisomycin resulted in a 3-fold induction of telomerase activity in FHIOSE 118 cells by 24 h (Fig. 2), demonstrating that JNK can both increase endogenous as well as induce telomerase activity. Anisomycin treatment did not affect cell viability, as determined by a cell proliferation assay (Fig. 2).

Endogenous JNK Activity Is Increased in Telomerase-positive Cells. Telomerase-negative FHIOSE 118 and telomerase-positive SW626 cells were compared for endogenous levels of members of the JNK signaling pathway (Fig. 3). After immunoprecipitation, cell lysates were probed with anti-phospho-SEK1, anti-phospho-JNK, and anti-phospho-c-Jun. Western blot analysis revealed that of the JNK signaling cascade proteins examined, FHIOSE 118 cells did not have elevated levels of phosphorylated proteins. In contrast, SW626 cells were found to have high levels of phosphorylated SEK1, JNK, and c-Jun. β-actin was used as a loading control. Several telomerase-positive ovarian cancer cells and telomerase-negative, nontumorigenic ovarian cell lines were also surveyed for endogenous levels of JNK, phosphorylated JNK, and telomerase activity. We found that telomerase-positive cell lines have elevated endogenous phospho-JNK levels when compared with telomerase-negative cell lines (Fig. 3).

JNK Plays a Role in Telomerase Regulation. To confirm a role for JNK in telomerase regulation, the telomerase-negative FHIOSE 118, NFHIOSE 80, and FHIOSE 1816-575 cells were transfected with GFP or JNK, whereas the highly telomerase-positive CaOV3 ovarian cancer cells were transfected with GFP and JIP (Fig. 4). The FHIOSE 118 cells were also cotransfected with JNK and the JNK inhibitor protein, JIP. FHIOSE 118 cells transfected with JNK exhibited an 8-fold induction of telomerase activity, whereas transfection with GFP or JNK + JIP did not induce detectable telomerase activity (Fig. 4). Furthermore, JIP was able to suppress JNK-induced telomerase activity in the FHIOSE 118 cells (Fig. 4). Successful transfections were confirmed by Western blot analysis (Fig. 4), and transfection efficiency in FHIOSE 118 cells was ~70% as visualized compared with GFP controls. To confirm the effect of JNK, two additional telomerase-negative cell lines, NFHIOSE 80 and FHIOSE 1816-575 cells, were also transfected with JNK (Fig. 4). Twenty-four h after transfection with JNK, NFHIOSE 80 cells exhibited a 5.75-fold increase in telomerase activity, and the FHIOSE 1816-575 cells showed a 3-fold increase. For NFHIOSE 80 and FHIOSE 1816-

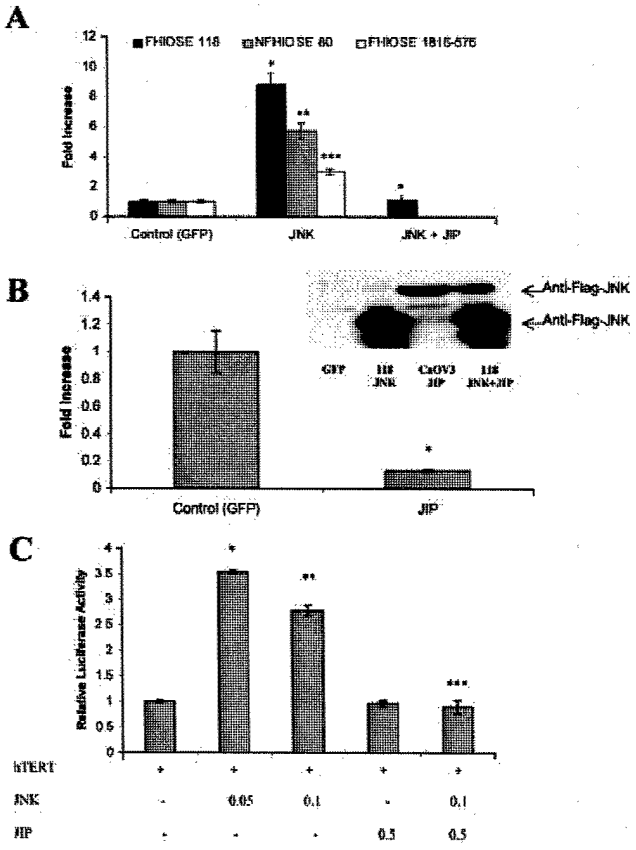

Fig. 4. JNK is capable of inducing telomerase activity by driving the hTERT promoter. the telomerase-negative FHIOSE 118, NFHIOSE 80, and FHIOSE 1816–575 cells were transfected with JNK or GFP as a control, and the FHIOSE 118 cells were also cotransfected with JNK + JIP. Cells were collected 24 h after transfection and assayed for telomerase activity. Telomerase levels are expressed as fold increase; SE. *, ≤ 0.02; **, ≤ 0.005; ***, ≤ 0.002. Successful transfection in FHIOSE 118 cells was confirmed by Western blot analysis ( ). the telomerase-positive CaOV3 cells were transfected with JIP or GFP, as a control. Cells were collected 24 h after transfection and assayed for telomerase activity. Telomerase levels are expressed as fold increase; SE. *, ≤ 0.008. Successful transfection was confirmed by Western blot analysis ( ). FHIOSE 118 cells were transfected with the hTERT promoter fused to a luciferase reporter gene alone or in combination with JNK ± JIP. Samples were collected 48 h after transfection and assayed for luciferase activity. Transfection efficiencies for all samples were normalized with β . Samples are expressed as fold increase; SE. *, ≤ 0.008; **, ≤ 0.05; ***, ≤ 0.003.

575 cells, transfection efficiencies were approximately 55 and 30%, respectively, as visualized compared with GFP controls. Transfection of JIP into CaOV3 cells resulted in a 10-fold decrease in telomerase activity (Fig. 4), confirming the role of JNK in the regulation of telomerase. For CaOV3 cells, transfection efficiency was ~35% as visualized compared with GFP controls. GFP did not affect telomerase activity (data not shown). Successful transfections were confirmed by Western blot analysis (Fig. 4).

JNK Is Capable of Activating the hTERT Promoter, and This Activation Can Be Inhibited by JIP. To clearly demonstrate the role of JNK in telomerase regulation, it was necessary to determine whether JNK was capable of activating transcription of a reporter gene fused to the hTERT promoter sequence (Fig. 4). Telomerase-negative FHIOSE 118 cells were transfected with either the hTERT promoter reporter construct ± JNK, ± JIP. Samples were collected 48 h after transfection, and luciferase activity was measured (Fig. 4). The hTERT promoter reporter was activated by as little as 0.05 μg of JNK, resulting in a 3.5-fold increase in reporter gene activity. In addition, JIP was able to abolish reporter activity when cotransfected with JNK, but alone, JIP did not induce reporter activity.

Discussion

Our study is the first to identify JNK as a regulator of telomerase. These data are in agreement with previous studies that suggested a connection between the PI 3-kinase pathway and telomerase activity. Although hTERT may have a consensus sequence for the Akt kinase, our attempts to induce telomerase activity in the OSE cell system with activated Akt were unsuccessful. This would suggest that Akt, the primary downstream target of PI 3-kinase, does not contribute to telomerase regulation in OSE cells. Instead, we showed that JNK, also a target of PI 3-kinase, is involved in telomerase regulation.

Although the hTERT promoter has been shown to respond to c-myc, many binding sites have been identified for several different transcription factors within the promoter region (17). This would suggest a complex mechanism for telomerase regulation that is probably cell type or tissue specific. However, we revealed that JNK alone was capable of activating transcription of the luciferase gene fused to the hTERT promoter sequence in our OSE cell system. Although it is not known precisely how JNK activates the hTERT promoter, it is possible that JNK-mediated phosphorylation and activation of c-Jun can lead to c-Jun binding to any of several AP1 sites present throughout the hTERT promoter (18). In support of this idea, Satoru and Inoue have demonstrated that loss of the AP1 site in the hTERT promoter significantly reduces hTERT expression and consequently, telomerase activity.

In summary, our study is the first to indicate that JNK can induce telomerase activity by transcriptional activation of hTERT. The initial extracellular signal may stimulate PI 3-kinase and cascade through SEK1, then JNK. JNK, in turn, could activate c-Jun, causing transcription of hTERT and subsequent activation of telomerase. Certainly, additional studies are warranted to completely delineate the signaling pathway for JNK-mediated telomerase regulation. These data will have a significant impact on the development of chemotherapeutic agents to target telomerase.

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